CHARACTERIZATION OF RAFFINOSE FAMILY OLIGOSACCHARIDES IN LENTIL SEEDS

A Thesis Submitted to the College of Graduate Studies and Research in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Department of Plant Sciences University of Saskatchewan, Saskatoon, Saskatchewan

By

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ABSTRACT

Raffinose family oligosaccharides (RFO) are major soluble carbohydrates of lentil (*Lens culinaris* Medik) seeds. When consumed by humans, RFO pass indigested through upper digestive tract as α-galactosidase enzyme required for RFO breakdown is not produced in humans. Consumption of lentils with high concentrations of RFO result in stomach discomfort, bloating and diarrhea due to bacterial fermentation of RFO in large intestine. This has lead to a relatively low use of lentils for human consumption. RFO are therefore considered anti-nutritional factors and development of lentil cultivars with reduced RFO concentrations is desired to improve lentil quality and consumption. To explore the possibility to develop lentil cultivars for low-RFO concentration, heritability of RFO trait and influence of environmental conditions on RFO must be known. In addition, RFO biosynthesis and accumulation in lentil seeds must be understood. However, very limited information is available on the above mentioned aspects of RFO in general and in lentil in particular. Therefore, the objectives of this study were: (1) to evaluate natural variation in RFO concentration and composition in commonly grown lentil cultivars and to determine the correlation between RFO concentration and other important seed constituents, (2) to investigate heritability and effect of environment on concentration and composition of lentil seed soluble carbohydrates, (3) to assess natural variation and diversity in RFO concentration in the genus *Lens*, (4) and to evaluate the association between galactinol synthase activity and accumulation of RFO in lentil seeds. Analysis of 22 lentil genotypes revealed significant ($P \leq 0.05$) variation in total starch, amyolse, protein, total RFO and seed weight and seed colour. Stachyose was the major RFO in all lentil genotypes followed by raffinose and verbascose. A significant ($P \leq 0.05$) inverse correlation was found between RFO and amylose concentration ($r = -0.34$); whereas RFO concentration and thousand seed weight correlated positively ($r = 0.35$). The analyses of variance of eleven cultivars grown at ten different environments showed that cultivar, environment and their interaction had significant effects on sugar concentration in lentil seeds. The high broad sense heritability of RFO ($h^2 = 0.85$) indicated that RFO concentration in lentil seeds is highly heritable and thus amenable to genetic improvement. An extensive evaluation of domesticated and wild species and subspecies of the genus *Lens* revealed significant ($P \leq 0.05$) variation and diversity in RFO concentration and composition of individual oligosaccharides. Higher Shannon-Weaver diversity

indices (SDI) for total RFO, raffinose and verbascose traits were observed in wild lentils compared to domesticated genotypes. *Lens ervoides* genotypes and some wild genotypes contained almost half the RFO concentration of cultivated lentils and therefore, wild genotypes may be useful for developing low-RFO lines. Higher verbascose and lower stachyose concentration was found in *Lens ervoides* genotypes, whereas higher raffinose and lower verbascose concentration is found in *Lens nigricans* genotypes.

Study of galactinol synthase activity in developing seeds with varying RFO concentration showed no clear association between galactinol synthase activity and RFO concentration. The sucrose and galactinol concentration of developing seeds were also not associated with total RFO concentration of lentil seeds. This finding suggests a non-regulatory role of galactinol synthase in RFO biosynthetic pathway in lentil seeds. Together, all these findings are not only significant to devise strategies to develop lentil cultivars with reduced RFO concentration but also for understanding RFO biosynthesis in lentil seeds.

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1 INTRODUCTION

1.1 Background

Lentil (*Lens culinaris* Medik.) is a nutritious pulse crop mainly used as a source of inexpensive and accessible high quality protein in human diets. Traditionally, lentil improvement has focused on the development of varieties with increased yield, resistance to various diseases and increased protein content. Though these objectives remain valid and important; recently lentil seed quality has drawn more attention for its health promoting qualities as well as some anti-nutritional effects associated with its consumption as a human food. The lens-shaped seeds of lentil are rich in proteins and its high lysine content provides a source of well balanced essential amino acids when consumed with cereal based-foods. Lentil is also a good source of carbohydrates, fiber, minerals and some vitamins. Lentil's fast cooking characteristics compared to other pulses also make it a desirable food crop. Nonetheless, the high quantities of raffinose family oligosaccharides (RFO) present in lentil seeds reduce its quality and are widely considered an impediment to increasing lentil consumption world wide (Delumen 1992; Messina 1999; Schneider 2002; Leterme 2002).

Sucrose and RFO are the major soluble carbohydrates of lentil seeds. RFO include raffinose, stachyose and verbascose. RFO are soluble carbohydrates ranked next to sucrose in their distribution in plants (French 1954; Keller and Pharr 1996), and can accumulate in storage tissues up to 16% of seed dry mass (Muzquiz et al 1999). However, more often the seed RFO concentration ranges from 2 - 10% (Horbowicz and Obendorf 1994; Loewus and Murthy 2000). RFO contain α-(1→6) glycosidic linkages which are indigestible by mammalian intestinal enzymes. RFO in food pass undigested through the stomach and are fermented by anaerobic bacteria in the lower intestine which produce gas causing flatus, discomfort and diarrhea in humans and monogastric animals (Price et al 1988). Large quantities of RFO in lentil are considered anti-nutritional and a reduction in RFO concentration in lentils is desired (Minorsky 2003). Various processing methods such as soaking (Vidal-Valverde et al 1993a, 1998, 2002), germination (Vidal-Valverde and Frias 1992), partial fermentation (Zamora and Fields 1979) and addition of α-galactosidase enzyme have been suggested to reduce RFO concentration in lentil and other pulses. These processing methods are either time consuming, expensive, result in

energy and nutrients loss or have consumer acceptability issues. Development of lentil cultivars with reduced RFO concentration is therefore important to improve lentil quality and increase human lentil consumption.

The seed constituents are affected by environment, genotype and environment×genotype interaction. Legume seed protein and starch content is known to vary with the genotype and the environmental conditions under which the crop is grown (Reichert and MacKenzie 1982; Wang and Daun 2006). Similarly, traits such as acid detergent fiber (ADF), neutral detergent fiber (NDF) and fat are more affected by genotype (Wang and Daun 2006). The effects of different environmental conditions on RFO concentration and composition in pulses under field conditions are largely unknown. It is therefore important to evaluate environmental effects on RFO concentration and to estimate RFO heritability in lentils to determine whether lentil seed constituents improvement through breeding can be successful. Furthermore, it is important to determine the association between RFO and several other important lentil seed constituents such as protein, starch and seed weight to assess and understand the effects of selection for low RFO trait on these seed components.

Crop improvement by breeding requires natural variation in the trait of interest. However, an extensive evaluation of variation and diversity of RFO concentration and composition in domesticated and wild lentils germplasm collections has not yet been carried out. Such assessment of variation in RFO concentration and composition of oligosaccharides will not only help in the selection of genotypes with reduced RFO concentration for lentil seed quality improvement programs but will also provide material for a thorough understanding of RFO biosynthesis in lentil seeds.

The biosynthetic pathway of RFO in plants involves five major enzymes including raffinose synthase, stachyose synthase, verbascose synthase, galactan-galactosyl trasferase and galactinol synthase. Galactinol synthase (EC 2.4.1.123) catalyzes the biosynthesis of galactinol, which is donor of galactosyl residues for RFO biosynthesis. Galactinol synthase has been reported as a key enzyme that may play a regulatory role in carbon partitioning between sucrose and RFO (Saravitz et al 1987). However, attempts to find a relationship between galactinol synthase activity and RFO concentration in seeds have produced mixed results. Therefore, analysis of galactinol synthase activity in lentil seeds with varying RFO concentration will help

to understand the relationship between galactinol synthase activity and accumulation of RFO in lentil seeds.

1.2 Research objectives

The objectives of this research program were:

1. To assess natural variation in RFO concentration and composition and to evaluate the association between RFO and other important seed constituents in selected lentil genotypes.
2. To determine the influence of environment on soluble carbohydrates and to estimate the broad sense heritability of RFO in lentil seeds.
3. To study natural variation in sucrose and RFO concentration and composition in the genus *Lens*.
4. To understand the relationship between galactinol synthase activity and RFO concentration in lentil seeds.

2 LITERATURE REVIEW

2.1 Introduction

2.1.1 Lentil origin and taxonomy

Lentil (*Lens culinaris* Medik.) (2n = 2x = 14) is an annual dicotyledonous plant in the family *Leguminosae* or *Fabaceae* (Fig 2.1). It was domesticated in southern Turkey northern Syria region (Cubero 1981; Cubero et al 2009) and was given the scientific name of *Lens culinaris* by Medikus in 1787. *Lens culinaris* ssp. *culinaris* is believed to originate from *Lens culinaris* ssp. *orientalis* (Boiss.) Hand.-Mazz. (Ladizinsky 1979; Cubero 1981; Ladizinsky et al 1984). Other species of the genus *Lens* Miller include *L. nigricans* (Bieb.) Godr., *L. ervoides* (Brign.) Grande., *L. lamottei* Czefr., *L. odemensis* Ladiz. and *L. tomentosus* Ladiz. (Cubero et al 2009).

2.1.2 Lentil morphology

Lentil is an indeterminate self pollinated plant that grows 15 to 75 cm in height (Muehlbauer et al 1985). The stem is generally herbaceous, light green and grows in a semi-erect pattern (Fig. 2.1). The plants vary from single stem to bushy and form dense or sparse stands. The pinnate leaves of lentils contain up to 14 sessile ovate leaflets, each about 1 - 4 cm long (Fig. 2.2c). Lentil flowers range from 4 to 8 mm in length and are white, pale purple or purple black (Fig 2.2a,b). Flowering is acropetal which results in pods of different maturity along the plant with the youngest pods at the top of stem. Lentil taproots penetrate 15 - 36 cm deep in soil and carry nodules that fix atmospheric nitrogen in a symbiotic relationship with Rhizobium and Vesicular- arbuscular-mycorrhiza species (Bala and Singh 1985). Self pollination occurs before the flowers open and pods becomes visible within 3 - 4 days. The mature pods are 1 - 2 cm long and usually contain two seeds. Based on seed size, lentil cultivars were in the past grouped as small-seeded (microsperma) and large-seeded (macrosperma); however, these groupings are no longer used (Saxena 2009). Nowadays, seed sizes are generally divided into small (3 - 5 mm), medium (5 - 6 mm) and large (6 - 9 mm) (Fig. 2.2d). The testa colour can be pink, yellow, green, dark green, grey, brown and black whereas cotyledons may be yellow, red or green. Black and dark brown spots can be found on testa in some genotypes (Fig 2.2d).

Figure 2.1. Lentil plant.

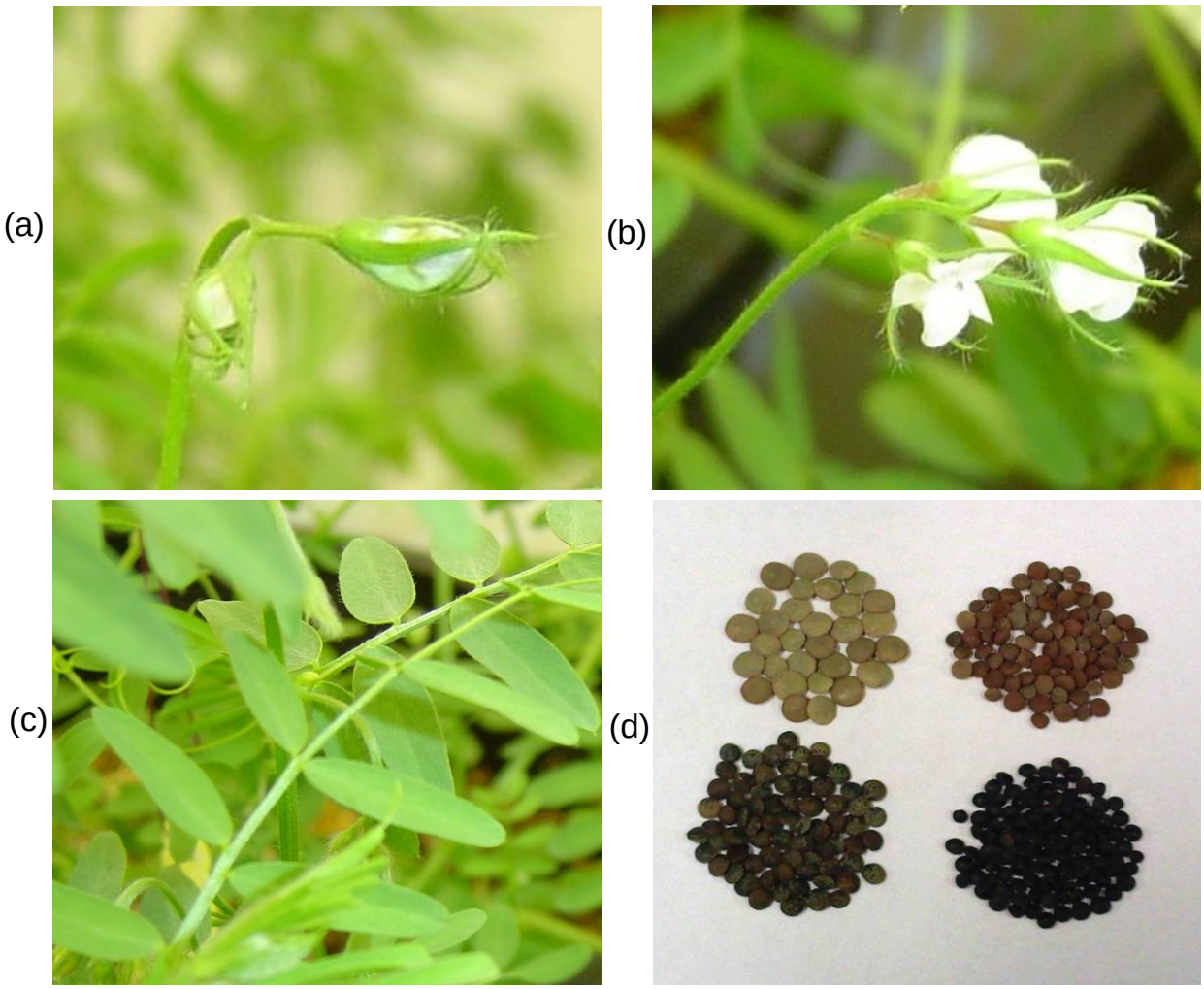

Figure 2.2. Picture showing (a) lentil flower bud (b) fully opened flower (c) mature herbaceous stem and leaves and (d) lentil seeds with different coat colour and spotting pattern.

2.1.3 Lentil adaptation and geographical distribution

Lentil is best adapted to the cooler temperate zones of the world and is predominantly grown in areas with an annual average rainfall of 300 to 400 mm (Sarker et al 2003). It can grow on a wide range of soil types, however, it is sensitive to water-logging, flooding and soils with a pH below 6.5 (Tang and Thomson 1996). Lentil yield, growing seasons and geographical distribution are largely determined by environmental factors such as temperature, amount and distribution of precipitation. For optimum yields, a lentil plant density of about 100 plants m^{-2} is recommended; however, suboptimum plant density may be compensated through development of lateral branches. In India, Pakistan, Nepal and Bangladesh, lentil is mainly grown as a winter crop on residual moisture from monsoon rains. In Saskatchewan, Canada, lentils are planted in the spring in brown and dark brown soil zones. Moisture from melting snow provides water for lentil crop establishment early in spring, whereas timely summer rains are needed to support plant growth for rest of season.

The genus *Lens* is mainly distributed in the Mediterranean region; however, individual species differ in their distribution. For example *Lens orientalis* is distributed from Turkey and Palestine to Uzbekistan. *Lens nigricans* on the other hand is mainly distributed from Palestine to Spain, Algeria and Morocco. Similarly, wild forms of *Lens ervoides* grow in Uganda and Ethiopia (Mishra et al 2007).

2.1.4 Lentil germplasm collections

The International Center for Agricultural Research in Dry Areas (ICARDA, Aleppo, Syria) has a global mandate for lentil improvement and holds the largest lentil germplasm collection in the world. The ICARDA seed collection is entirely *ex situ* as seed and consists of 10,800 genotypes. Land races or cultivars collected from 70 different countries dominate the collection (8,860), followed by breeding lines (1,373), and wild accessions (583) from 24 different countries (Furman et al 2009) (Fig. 2.3). Almost half of the collection (48%) comes from a region spanning Central and West Asia and North Africa, which is regarded as lentil's centre of origin and primary diversity (Zohary and Hopf 1988; Ferguson and Erskine 2001). A

quarter of the ICARDA germplasm collection is from South Asia and the remaining 25% from the rest of the world.

In addition to ICARDA collection, the lentil collections are located within the Australian Temperate Field Crops Collection (5,250 genotypes), USDA (2,797 genotypes), All-Russian Scientific Research Institute of Plant Industry collection (2,396 genotypes) and National Board of Plant Genetic Resource of India Collection (2,212 genotypes) (Diwiedi et al 2006).

2.1.4.1 Core collection

A core germplasm collection is a subset of a large collection that generally contains about 10% of the genotypes and adequately represents the genetic variability and diversity of the entire collection (Frankel and Brown 1984; Brown 1989). A lentil core or composite collection comprising 1,000 genotypes representing landraces, wild relatives and elite germplasm and cultivars was developed from the ICARDA germplasm collection (Furman 2006; Furman et al 2009). For creating this collection a set of 7,345 cultivated lentil genotypes from 65 different countries (12 geographic regions) and 238 wild *Lens* genotypes from 12 different countries were selected from ICARDA collection. The wild lentil genotypes are represented by the three putative progenitor species i.e. *L. culinaris* ssp. *orientalis*, *L. odemensis* and *L. tomentosus*. The cultivated genotypes in the germplasm collection set are separated into four data sets based on different years of evaluation and within each of these sets, by geographic location/region, resulting in a total of 50 data sets. These 50 data sets have undergone a hierarchical cluster analysis based on 12 phenological and agronomic characteristics. Approximately 10% of the genotypes in each cluster within a set were selected randomly and included in the ICARDA lentil core collection (Furman 2006). The lentil core collection thus obtained is representative of the collection set with similar Shannon-Weaver diversity indices for morphological traits for both core collection and the large set from which the core collection was derived (Furman 2006). Similarly, another lentil core collection with 384 genotypes has been developed by USDA Agricultural Research Service based on the genotypes country of origin (Simon and Hannan 1995). To reduce the size of core collections, mini core collections have been selected for some legumes. The mini core consist of about 10% of the genotypes of core collection or 1% of the entire collection but still represents the diversity of the entire core collection (Upadhyaya and Ortiz 2001). A mini core collection for lentil, however, has not been developed.

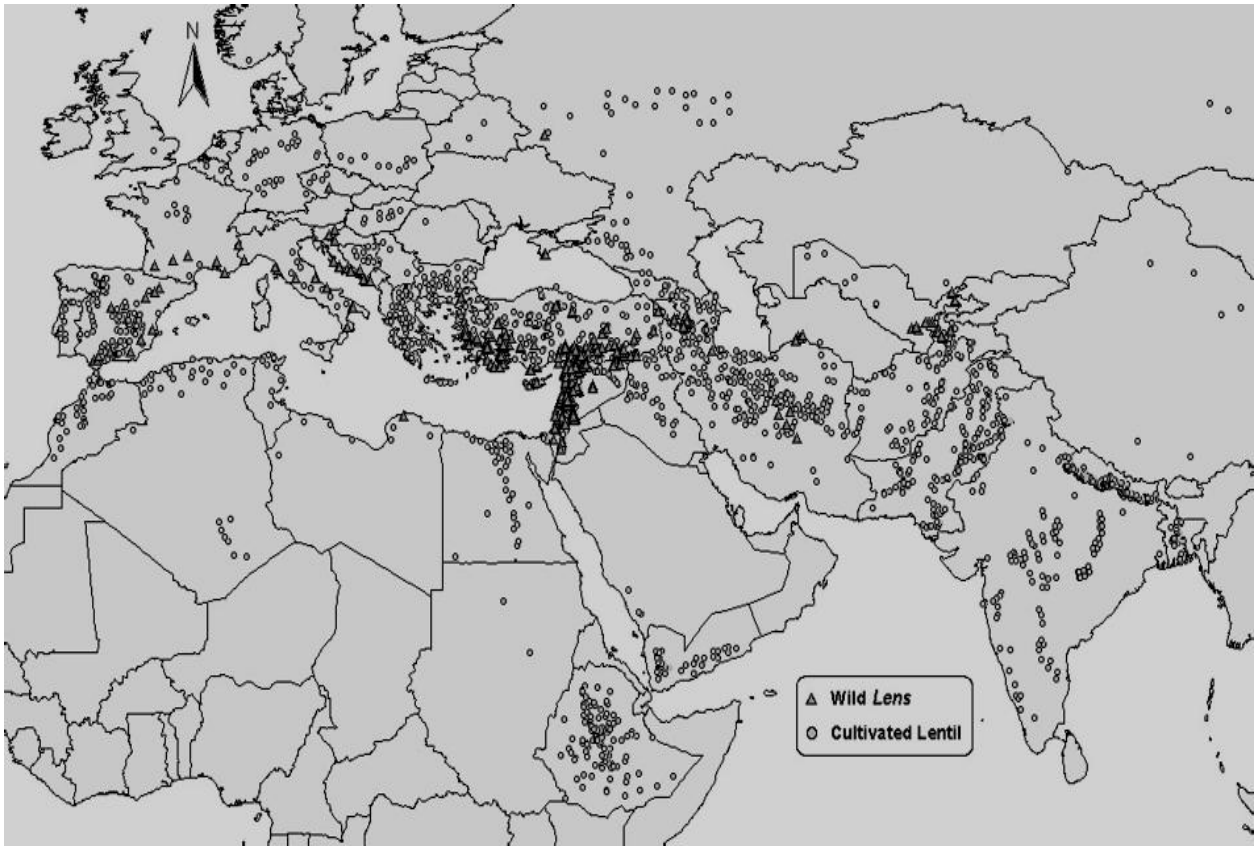

Figure 2.3. Geographical origin of ICARDA lentil germplasm collection.

2.1.5 Lentil utilization

2.1.5.1 Use as food and effects on human health

The most valuable part of the lentil crop is seed which is primarily used as human food. Lentils are mostly used in split form as dhal or as flour for making soups, stews, bread and cakes (Williams and Singh 1988; Aw-Hassan et al 2003). Lentil products are mostly consumed in South Asia, Middle East and the Mediterranean region. Lentil is often used in combination with rice in India and Middle East, whereas lentil soup is preferred in Europe and North and South America.

The consumption of pulses and lentil is associated with several health benefits including the control and prevention of diabetes (Jacobs and Gallaher 2004), cardiovascular disease (Hu 2003), osteoporosis and hypertension (Philanto and Korhonen 2003), colon cancer, and gastrointestinal disorders (Chibbar et al 2010). A report by Flight and Clifton (2006) estimated that consumption of legumes may result in a 22% and 11% lower risk of coronary heart disease and cardiovascular disease, respectively. Similarly, Bazzano et al (2001) reported a significant and inverse association between coronary heart cardiovascular disease and use of pulses in human diet. Many of the beneficial effects of lentil consumption on human health are due to lentil's low fat, high protein and high dietary fiber and relatively high concentrations of resistant starch which contribute to lentil's low glycemic index (GI). GI is a scale that ranks foods by how much they raise blood glucose levels upon consumption (Jenkins et al 1982; Jenkins 2007). Low GI foods (< 55) are recommended, because increased blood glucose and insulin levels from higher GI foods may result in obesity, type 2 diabetes and cardiovascular disease (Rizkalla et al 2002; Brand-Miller 2007). Chung et al (2008) using *in vitro* starch digestion assay compared the expected glycemic index (EGI) of lentil, pea (*Pisum sativum* L.) and chickpea (*Cicer arietinum* L.) and reported a lower EGI of 41.4 - 41.5 for lentil varieties compared to EGI of 44.4 - 38.9 and 48.9 - 56.1 for pea and chickpea cultivars, respectively. Lentil starch also has the lowest concentration of rapidly digestible starch (RDS: 7.6 - 7.8%) and the highest concentration of resistant starch (RS: 14.4 - 14.9%) when compared to pea (RDS: 9.2 - 10.7%; RS: 13.3 - 14.7%) and chickpea (RDS: 9.4 - 12.4%; RS: 3.1 - 6.4%) starches (Chung et al 2008). The concentration of rapidly digestible starch and resistant starch in food affect food digestibility and glycemic index, which have effects on human health.

2.1.5.2 Use in crop rotations, green manure crop and animal feed

Lentil and other legume crops are used in crop rotations to improve soil fertility and texture, thus increasing yields of subsequent crops (Wright, 1990, Gan et al 2003; Miller et al 2003). The positive effects of legume/cereal crop rotations are due to increased soil nitrogen content (Campbell et al 1992; Gan et al 2003), elevated soil moisture levels (Miller et al 2003) and disease suppression (Stevenson and van Kessel 1996). The use of lentil as a green manure crop increases soil nitrogen and carbon levels, provides protection against erosion and improves soil quality compared with the traditional fallow-wheat cropping system (Biederbeck et al 2005). The by-products of lentils such as leaves, stems, and bran also have a use as green manure or livestock feed (Yadav et al 2007).

2.1.6 Lentil production

Lentil ranked sixth among pulse crops in the world after dry bean, pea, chickpea, faba bean and cowpea in terms of production volume in the years 2003-2006 (FAO 2008, www.fao.org). World lentil production in 2008 was 3.9 million metric tonnes (FAO 2008) and average yields for 2004-2006 period was 950 kg/ha (Erskine 2009). The major lentil producing countries of the world include India, Canada, Turkey, Syria, Australia, Nepal and United States, (http://www.agriculture.gov.sk.ca). Lentil was introduced in Canada in 1969 and has become an important pulse crop in western Canada (Table 2.1). Most of Canadian lentils are grown in Saskatchewan where lentil cultivated area and production has steadily increased over the years. Canada is now one of the major lentil producing countries and a leading exporting country of the world. In 2010-11, Canada lentil production and exports are estimated at 1.55 and 1.3 million metric tonnes respectively (Agriculture and Agri-Food Canada, http://www.agr.gc.ca/). The importance of lentil in Saskatchewan agriculture is expected to continue due to an increased demand for Canadian lentils in the international market.

2.2 Lentil seed composition

Lentil has played an important role in human nutrition for centuries. It is a good source of carbohydrates, protein, most essential minerals and several vitamins. Lentil seed chemical constituents are are briefly discussed.

Table 2.1. Production overview of pulse crops in Western Canada.

Province	Harvested area (thousand hectares)		Production (thousand tonnes)		Yield (Kg/ha)		Mean production (thousand tonnes)
	2007	2008	2007	2008	2007	2008	1998 - 2007
			Lentil (*Lens culinaris)*				
Manitoba	-	-	-	-	-	-	5
Saskatchewan	534	631	674	920	1250	1460	708
Alberta	-	-	-	-	-	-	9
Total	534	631	674	920	1250	1460	718
			Pea (*Pisum sativum)*				
Manitoba	39	44	98	108	2540	2420	138
Saskatchewan	1164	1255	2310	2732	1990	2180	1855
Alberta	241	283	528	731	2190	2580	539
Total	1443	1582	2935	3571	2300	2300	2532
			Chickpea (*Cicer arietinum)*				
Manitoba	-	-	-	-	-	-	-
Saskatchewan	154	42	198	67	1290	1580	174
Alberta	20	-	27		1320	-	14
Total	174	42	225	67	1290	1580	189
			Common bean (*Phaseolus vulgaris)*				
Manitoba	26	22	43	36	1640	1630	67
Saskatchewan	-	-	-	-	-	-	-
Alberta	-	-	-	-	-	-	-
Total	26	22	43	36	1640	1630	67

Source: Statistics Canada (www.statcan.gc.ca), Field Crop Reporting Series, 1998-2008

2.2.1 Protein and amino acids

Lentil like all other pulses contains almost twice the amount of proteins as compared to cereal grains, most root crops, fruits and vegetables. The protein content of lentil seeds ranges from about 21 to 31% (Bhatty 1984; Combe et al 1991; Kavas and Nehir 1992; Porres et al 2002; Amjad et al 2003; Iqbal et al 2006). Within lentil seeds, the cotyledon, embryo and seed coat contains 80 - 90%, 2% and 8 - 20% protein, respectively (Adsule et al 1989; Cuadrado et al 2002). Lentil proteins are generally stored in protein bodies (Wang et al 2003) and mostly consist of salt- soluble globulins and water-soluble albumins. The major globulins in lentils are legumins (11S) (44.8%) and vicilins (7S) (4.2%). Legumins contain higher amounts of sulphur-containing amino acids (methionine and cysteine) compared to the vicilins proteins (Bulter 1982). Albumins constitute 16.8% of lentil proteins and are primarily composed of enzymes and protease inhibitors proteins and are contain high levels of cysteine and methionine. Glutelins and prolamins which are soluble in dilute acid/alkali detergents and alcohol constitute 11.2% and 3.5% of lentil proteins, respectively (Osborne 1924; Gupta and Dhillon 1993). The relative concentration of albumin to globulin and legumins to vicilins affect the amino acid profile and protein quality (Bulter 1982). Ratios of 1:3 for albumin to globulin and 10.5:1 for legumins to vicilins have been reported for lentil (Gupta and Dhillon 1993). Lentil proteins provide a good amino acid profile for human diet where most essential amino acids are well represented. The amino acids lysine, leucine, isoleucine, phenylalanine, histidine, tyrosine, threonine and valine concentrations are present in higher quantities than FAO/WHO (1991) recommendations for human diet. However, lentil is deficient in tryptophan and sulphur-containing essential amino acids methionine and cysteine (Peace et al 1988; Wang and Daun 2006) (Table 2.2).

Legumes such as peanuts and soybean are often implicated in human food allergic reactions, whereas lentil allergies are less frequent (Pascual et al 1999). Two allergens designated as Len c1 (12 to 16 kDa) corresponding to γ-vicilin subunits and Len c2 (66 kDa), seed-specific biotinylated protein, have been identified in boiled lentils (Sánchez-Monge et al 2000).

Table 2.2. Amino acid composition of lentil.

Essential Amino Acids	Concentration		FAO/WHO recommendation
	g/16 g N[a]	g/16 g N[b]	g/16 g N
Histidine (His)	1.3 - 4.0	2.6 - 3.3	1.9
Isoleucine (Ile)	2.6 - 9.6	4.4 - 5.5	2.8
Leucine (Leu)	5.7 - 15.9	6.8 - 8.7	6.6
Lysine (Lys)	4.0 - 12.6	6.3 - 8.2	5.8
Threonine (Thr)	2.5 - 7.6	3.4 - 4.4	3.4
Tryptophane (Trp)	ND - 2.6	0.6 - 0.9	1.1
Valine (Val)	3.3 - 11.6	4.7 - 6.1	3.5
Phenylalanine (Phe)	3.6 - 10.6		
Methionine (Met) + Cysteine (Cys)	0.8 - 1.6	2.2 - 4.2	3.5
Arginine (Arg)	3.9 - 14.0	6.7 - 8.8	
Non-Essential Amino Acids			
Alanine (Ala)	2.4 - 39.8	3.9 - 5.0	
Aspartate (Asp) + Asparagine (Asn)	9.3 - 26.1	10.6 - 13.8	
Glutamate (Glu) + Glutamine (Gln)	12.8 - 42.3	14.4 - 18.3	
Glycine (Gly)	3.3 - 12.7	3.7 - 4.8	
Cysteine (Cys)	0.4 - 1.5		
Proline (Pro)	1.2 - 11.4	3.6 - 4.6	
Serine (Ser)	2.9 - 15.6	3.8 - 4.4	
Tyrosine (Tyr) + Phenylalanine (Phe)	1.1 - 7.5	7.4 - 9.4	6.3

Adapted from Wang and Daun (2006), Grusak (2009) and Boye et al (2010).

(a) Concentration in lentils and (b) concentration in Canadian varieties.

2.2.2 Carbohydrate

Carbohydrates are the major constituents of lentil seeds (Table 2.3). Carbohydrates can be defined as polyhydroxy aldehydes or ketones or substances that produce such compounds on hydrolysis (Nelson and Cox 2005). They can be grouped into monosaccharides, disaccharides, oligosaccharides and polysaccharides based on the number of polyhydroxy aldehyde or ketone units. Monosaccharides (derived from Greek, monos: single, sakcharon: sugar) are the simplest sugars and the basic units of more complex carbohydrates. They consist of single polyhydroxy aldehyde or ketone unit. Disaccharides consist of two monosaccharides whereas oligosaccharides are composed of 3 - 20 monosaccharides residues joined by glycosidic bonds. Polysaccharides are polymers of monosaccharide residues containing more than 20 to hundreds or thousands of monosaccharide residues (Nelson and Cox 2005).

2.2.2.1 Monosaccharides and disaccharides

Monosaccharides such as glucose and fructose are present in small quantities in lentil seeds. The glucose and fructose concentrations of lentil seeds vary from non-detectible to 0.04 g and from 0.01 - 0.2 g 100 g^{-1}dry matter, respectively (Vidal-Valverde et al 1993a, 1993b; Frias et al 1994, 1995, 1996). Sucrose is the major disaccharide in lentil seeds with a concentration ranging from 1.1 - 3.0 g 100 g^{-1} dry matter (Vidal-Valverde et al 1993a; Frias et al 1994, 1996; Wang and Daun 2006). The concentration of the disaccharide maltose ranges from 0.05 - 0.33 g 100 g^{-1} dry matter (Table 2.3) (Grusak 2009).

2.2.2.2 Oligosaccharides and cyclitols

Alpha-galactosides of the raffinose family e.g. raffinose, stachyose and verbascose (RFO) are the most abundant oligosaccharides in lentil seeds. RFO concentration in lentil seeds range from 1.8 - 6.8 g 100 g^{-1}dry matter (Frias et al 1994; 1996; Wang and Daun 2006; Vidal-Valverde et al 1993a). The stachyose, raffinose and verbascose content of lentil seeds have been reported to range from 1.1 - 3.1, 0.2 - 1.5 and from non-detectable to 3.1 g 100 g^{-1}dry matter, respectively (Vidal-Valverde et al 1993a, 1993b; Frias et al 1994, 1995, 1996; El-Adawy et al 2003; Wang and Daun 2006). Seeds of legume species also accumulate galactosides of myo-inositol isomers (or methylated ethers), collectively named galactosyl cyclitols. Galactopinitol A, ciceritol,

galactopinitol B and fagopyritol B1 are the major galactosyl cyclitols in lentil, soybean, and chickpea (Lahuta et al 2010). The ciceritol concentration in lentil seeds is much lower than the RFO concentration and ranges from 0.24 - 1.99 g 100 g^{-1} dry matter (Vidal-Valverde et al 1993a, 1993b; Frias et al 1995, 1996). Verbascose is the only member that remains at undetectable levels in some lentil genotypes.

2.2.2.3 Polysaccharides

2.2.2.3.1 Starch

The predominant carbohydrate of lentil seeds is starch, for which the concentration varies from 34.7 - 65.0 g 100 g^{-1}dry matter (Frias et al 1994; Wang and Daun 2006). Starch is stored in granules within amyloplasts of plant cells and is the primary source of available energy from lentil seeds. Starch is composed of amylose and amylopectin. Amylose is a linear polymer of α-D-glucose units with α-(1→4) linkages and a few α-(1→6) α-D-glucose branches (Kennedy et al 1983). Amylopectin is a highly and regularly branched molecule consisting of a main α-(1→4) α-D-glucose chain with α-(1→6) α-D-glucose branches. The amylose content of lentil seeds ranges from 20.0 - 45.5 g 100 g^{-1} starch (Urbano et al 2007; Grusak 2009) and the relative proportion of amylose and amylopectin gives characteristic physiochemical properties to starches (Hoover and Ratnayake 2002).

2.2.2.3.2 Non starch polysaccharides

The non starch polysaccharides of lentil seed include cellulose and hemicellulose, which are the major constituents of insoluble dietary fiber. Both polymers are structural component of primary cell walls in plants. The concentration of cellulose and hemicellulose in lentil seeds range from 4.1 - 5.33 g, and 6.0 - 15.74 g 100 g^{-1} (Vidal-Valverde and Frias 1991; Vidal-Valverde et al 1992), respectively.

2.2.3 Minerals, vitamins, lipids and fatty acids

Lentils are a good source of several essential macronutrients (K, P, and Mg), micronutrients (Fe, Zn, Cu, Mn) and trace elements (Cr, Co, Se, and Mo) in the human diet (Table 2.4).

Table 2.3. Carbohydrate concentrations in lentil seeds

Carbohydrate	Concentration g/100 g dry matter
Total carbohydrates	43.0 - 70.0
Total soluble sugars	2.3 - 8.9
Total available sugars	1.1 - 3.2
Glucose	ND - 0.04
Fructose	0.01 - 0.2
Sucrose	1.1 - 3.0
Maltose	0.05 - 0.33
Total α-galactosides	1.8 - 6.8
Raffinose	0.2 - 1.5
Stachyose	1.1 - 3.1
Verbascose	ND - 1.4
Ciceritol	0.2 - 2.0
Starch	34.7 - 65.0
Cellulose	4.1 - 5.3
Hemicellulose	6.0 - 15.7

Values derived from the following references: Vidal-Valverde and Frias 1991; Vidal-Valverde et al 1993a; Frias et al 1994, 1996; Ereifej and Haddad 2001; El-Adawy et al 2003; Wang and Daun 2006; Cai et al 2002, Urbano et al 2007; Grusak 2009.
ND: Not detectable

Lentil mineral composition and profile may be assessed in terms of lentil contribution towards fulfilling the required Recommended Dietary Allowance (RDA) of minerals in a single serving. The RDAs are intake levels of essential nutrients considered adequate to meet the needs of healthy individuals (Grusak 2009). Based on RDA of essential minerals, lentils may be regarded as a good source of copper, iron, zinc and manganese and a poor source of calcium (Grusak 2009, Table 2.4). Most of the phosphorus in lentil seeds is stored as phytic acid, which ranges in concentration from 0.15 - 2.34 g 100 g^{-1} (El-Adawy et al 2003; Rehman and Shah 2005; Wang and Daun 2006). Human and monogastric animals lack phytase which is required to breakdown phytic acid and are unable to use this compound as a source of available phosphorus. Phytic acid may also interfere with the utilization of proteins and minerals due to formation of phytic acid-protein and phytic acid-mineral-protein complexes, which reduce protein and mineral absorption in small intestine (Cheryan 1980; Reddy et al 1982). Therefore, lentil cultivars with reduced phytic acid concentration and increased inorganic phosphorus concentration are desired.

Lentils are a good source of several of the water-soluble vitamins required in human diet (Table 2.4). Relative to maximum RDA, lentils provide good levels of folic acid, pantothenic acid, pyridoxin and thiamin. However, the fat-soluble vitamin concentrations of lentil seeds are low when compared to RDA (Table 2.4). The crude fat concentration of lentil seeds ranges from 0.7 - 4.3 g 100 g^{-1}dry matter (Porres et al 2002; Iqbal et al 2006; Wang and Daun 2006). Linoleic acid (C18:2), oleic acid (C18:1), linolenic acid (C18:3), and palmitic acid (C16:0) are major fatty acids in green lentils with concentration ranging form 41.0 - 46.1 g, 17.0 - 25.6 g, 11.9 - 16.2 g and 10.8 - 15.4 g 100 g^{-1}crude fat, respectively (Canadian Grain Commission 2004).

2.3 Raffinose family oligosaccharides (RFO)

The RFO concentration and composition in pulses, fruits and vegetables have been determined in several studies (Vidal-Valverde et al 1993a, 1993b; Frias et al 1994, 1995; Wang and Daun 2006). These reports show considerable variation for RFO and sugar concentrations measured in pulses. The soluble carbohydrates of soybean (*Glycine max* L.) seeds constitute about 15.0 g 100 g^{-1}seed dry mass mostly as sucrose and RFO, and only small quantities of galactosyl cyclitols such as galactinol, fagopyritols, and galactopinitols (Hsu et al 1973; Schweizer and Horman 1981; Obendorf et al 1998).

Table 2.4. Mineral and vitamin composition of lentil seed.

	Concentration (mg 100 g^{-1} dry matter)	Maximum RDA (mg)	Cooked lentils mg, 200 g^{-1}
Minerals			
Calcium (Ca)	33 - 210	1200	38
Magnesium (Mg)	13 - 220	420	72
Phosphorus (P)	239 - 725	700	360
Potassium (K)	240 - 1440	4700	738
Iron (Fe)	3.1 - 14.6	18	6.6
Zinc (Zn)	2.5 - 4.4	11	2.6
Manganese (Mn)	1.3 - 5.4	2.3	1.0
Copper (Cu)	0.9 - 10.0	0.9	0.5
Selenium (Se)	0.009 - 0.012	0.06	0.01
Sodium (Na)	0.4 - 180		
Boron (B)	0.6 - 1.1		
Chromium (Cr)	0.03		
Molybdenum (Mo)	0.08 - 0.22		
Water soluble vitamins			
Vitamin C (Ascorbic acid)	ND - 7.7	90.0	3.0
Thiamin (vitamin B_1)	0.1 - 0.9	1.2	0.3
Riboflavin (vitamin B_2)	0.03 - 0.5	1.3	0.15
Niacin (vitamin B_3)	0.6 - 3.6	16.0	2.12
Pyridoxine (vitamin B_6)	0.55 - 0.60	1.7	0.36
Biotin (vitamin B_7)	0 - 0.132	0.03	0.11
Pantothenic acid (vitamin B_5)	0.4 - 2.4	5.0	1.28
Folic acid (vitamin B_9)	0.03 - 1.5	0.4	0.4
Fat soluble vitamins			
Vitamin A	0.002 - 0.0034	0.9	0.02
Vitamin K	0.0056	0.12	0.003

Values derived from the following references: Pores et al 2003, 2004; Wang and Daun 2006; Iqbal et al 2006; Urbano et al 2007; Grusak 2009
ND = Non detectable

The total α-galactosides concentration in soybean is 6.0 - 8.0 g 100 g^{-1} with stachyose as the major RFO (Reddy et al 1984; Hollung et al 2005; Sosulski et al 1982). Considerable variation in the total α-galactoside concentration and composition exists for pea (*Pisum sativum*) cultivars. In an analysis of 18 pea genotypes sucrose, raffinose, stachyose, verbascose and total α-galactoside concentration ranged from 1.16 - 2.54 g, 0.41 - 1.03 g, 1.07 - 2.67 g, 0.0 - 2.67 g and 2.26 - 6.34 g 100 g^{-1}dry matter, respectively (Vidal-Valverde et al 2003). A wider range of stachyose (0.7 - 4.1 g 100 g^{-1}dry matter) and verbascose (non-detectable levels - 3.1 g 100 g^{-1}dry matter) concentration in pea seeds was reported by Jones et al (1999). Some pea cultivars have stachyose as the major oligosaccharide, whereas verbascose is predominant in other cultivars (Karner et al 2004).

Chickpeas (*Cicer arietinum* L.) contain high quantities of RFO and galactosyl cyclitols. The concentrations of raffinose, stachyose and verbascose in chickpeas are about 1.5 g, 2.6 g and 0.2 g 100 g^{-1} dry matter, respectively (Alajaji and El-Adawy 2006). A study of three chickpea cultivars found the raffinose concentration to range from 1.9 - 2.8 g 100 g^{-1} dry matter, whereas the combined stachyose plus verbascose concentration was 0.9 - 1.7 g 100 g^{-1} dry matter (Mulimani and Ramalingam 1997).

2.3.1 Prebiotics

Humans and other monogastric animals lack the α-galactosidase enzyme required for breakdown of α-(1→6) linkages present between the galactose residues in RFO. As a result, RFO pass undigested through the stomach and lower intestine and are subsequently fermented in the colon by anaerobic bacteria (Saini and Knights 1984). RFO together with other non-digestible oligosaccharides, such as fructo-oligosaccharides and resistant starch, are considered prebiotics. Prebiotics are by definition any "nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson and Roberfroid 1995). The fermentation products of these oligosaccharides include carbon dioxide, hydrogen, methane and short chain fatty acids (SCFA) such as acetic, propionic and butyric acids (Ruppin et al 1980; Saini and Knights 1984). The amount and type of fermentation products depend on the carbohydrate. Raffinose, barley beta-glucans, oligofructose and some resistant starches produce butyric acid upon fermentation *in vitro* (Casterline et al 1997; Karppinen et al 2000) and *in vivo* when consumed by rats (Berggren

et al 1993; Mathers et al 1997). Other carbohydrates such as arabinogalactans produce propionic acid (Berggren et al 1993; Edwards 1993).

The health promoting effects of RFO as prebiotics include stimulation of the growth of beneficial bacteria such as bifidobacteria. The RFO chemical structure allows their consumption by a limited number of bacteria and thus reduces pathogenic organisms in the colon (Roberfroid 2002). The SCFA produced as a result of fermentation also play a role in the prevention and treatment of cancer and ulcerative colitis (Whitehead et al 1986; Gamet et al 1992; Scheppach et al 1995; Cummings 1997). Butyrate acts as an energy source for epithelial cells (Bugaut and Bentenjac 1993) and may prevent colon cancer by promoting cell differentiation (Kim et al 1982). Propionic acid reduces plasma cholesterol concentration by inhibiting cholesterol synthesis from acetic acid in the liver (Wolever et al 1991), improves colonic muscular contraction, and thus reduces constipation (Yajima 1985). Oligosaccharides in food also positively affect the availability and absorption of certain minerals in colon and large bowl. The osmotic effect of oligosaccharides transfers water into large bowel and increases the volume in which minerals can dissolve. SCFA reduce pH in colon and increase availability and absorption of minerals particularly calcium and magnesium (Yanahira et al 1997) and consequently help in prevention of osteoporosis and anemia (Scholz-Ahrens and Schrezenmeir 2002).

2.3.2 Anti-nutritional factors

Although RFO have positive effects on human health, the intake of large quantities of lentils leads to negative effects such as stomach discomfort, flatulence, bloating and diarrhea. These negative effects are mainly due to RFO fermentation in colon and are major hindrances in increasing the intake of pulses in food. The degree of these negative effects, however, depends on the amount of oligosaccharides consumed and individual's sensitivity to α-galactosides (Calloway et al 1971; Price et al 1988; Roberfroid et al 1998). For most people, a consumption of 3 g of α-galactosides per day (50 g lentils) does not produce any negative effects. Higher daily intake, are expected to cause problems associated with RFO consumption.

A reduction in α-galactoside concentration and an increase in metabolizable energy available from seed meals for livestock is desired by the feed industry. Diets containing 5.3% α-galactosides on a dry weight basis show a 20% reduction in net energy compared to a diet containing only 1% of α-galactosides (Coon et al 1990). The carbohydrates of soybean meal,

which is extensively used as animal feed, is mainly comprised of sucrose, raffinose and stachyose (Hymowitz and Collins 1974). Soybean α-galactosides create digestive disturbances in baby pigs, dogs and chickens and therefore, its use in animal feeds is kept at low level (Hartwig et al 1997). Reduction in RFO in soybean seeds will increase its use as animal feed and help in creating a more efficient feed source.

2.3.3 Processing methods for reduction of RFO in food

Various processing methods such as soaking, partial germination, partial fermentation and addition of enzymes have been used to reduce the α-galactoside content of pulses and improve their nutritional quality.

2.3.3.1 Soaking

Soaking pulses overnight and then discarding the water results in a considerable reduction in α-galactoside concentration. A 26% and 28% reduction in stachyose and raffinose concentration respectively, can be achieved by soaking cowpea flour for 16 hr (Somiari et al 1993). Soaking of chickpeas reduces α-galactosides concentration by 16 - 27% (Frias et al 2000). The efficiency of soaking process can be increased by adding sodium bicarbonate (Ibrahim 2002), increasing soaking time (Abdel-Gawad 1993), temperature or seed to water ratio (Jood et al 1985; Vijayakumari et al 1996).

2.3.3.2 Partial germination

RFO reserves in seeds are hydrolyzed by α-galactosidases during germination to support growth. Therefore, partial germination may be used to reduce α-galactosides concentration in pulses. During germination RFO in the axis are hydrolyzed before RFO in cotyledons in soybean, pea, and lupin seeds. The RFO in axis are broken down within two days compared to 4 - 6 days in cotyledons (Górecki et al 1997; Lahuta et al 1997). Germination of pigeon peas (*Cajanus cajan*) for four days at 20 °C in dark sharply decreases α-galactosides by 83% (Torres et al 2007). Three days germination of lentils reduces RFO concentration by 18 - 40% (Frias et al 1996) and upon six days germination, the stachyose, sucrose and ciceritol concentration are at undetectable levels (Urbano et al 1995). Similarly, drastic reductions in RFO contents are also observed for several non-conventional legumes such as cowpea (*Vigna unguiculata*), jack bean

(*Canavalia ensiformis*), mucuna (*Stizolobium niveum*) and dolichos (*Lablab purpureus*) after germination (Martin-Cabrejas et al 2008).

2.3.3.3 Natural fermentation

Natural fermentation is a processing method that reduces RFO concentration, increases the bioavailability of carbohydrates and improves aroma, texture and digestibility of proteins (Kazanas and Fields 1981; Salunke et al 2000). Natural fermentation of common peas (*Phaseolus vulgaris*) for 48 and 96 hr reduces the stachyose concentration by 72 and 95%, respectively (Granito et al 2003). In pigeon pea (*Cajanus cajan* (L.) Millsp.), seed fermentation reduces α-galactoside concentration, phytic acid and trypsin inhibitor activity by 82%, 48%, and 39%, respectively. More recently, Khattab and Arntfield (2009) observed that fermentation can reduce α-galactoside concentration by 71 - 72% in various pulses. A similar reduction of α-galactosides concentration along with significant reductions in protein and starch concentration is seen in cowpeas (*Vigna sinensis*) upon fermentation (Granito et al 2005). However, fermentation has several negative effects as the protein, dietary fiber, calcium, vitamin B2, vitamin E, antioxidant capacity, soluble dietary fiber, Na, K, Mg, and Zn concentration are also reduced (Torres et al 2006).

2.3.3.4 Addition of *α*- galactosidase

Alpha-galactosidase catalyzes the hydrolysis of terminal non-reducing α-(1→6) linked galactosyl residues from a wide range of galacto-oligosaccharides and polysaccharides (Naumoff 2004) and can be used to remove α-galactosides from legumes. Alpha-galactosidases are produced by various microorganisms including fungi, yeasts and bacteria. Fungal α-galactosidases are effective in RFO removal from soya milk and soya whey (Cruz et al 1981; Cruz and Park 1982), cowpea (Somiari and Balogh 1993) and chickpea flours (Mansour and Khalil 1998).

All these processing methods/approaches for reducing the RFO concentration in foods have some limitations. They are either time consuming, expensive or result in loss of energy and nutrients. Development of low RFO varieties in lentils and in other pulses is therefore desired.

2.3.4 Physiological roles in plants

RFO accumulate in seeds, stems, leaves, roots, and tubers of plants (Avigad and Dey 1997). The highest RFO concentration is found in seeds, where RFO play important roles during seed germination, acquisition of desiccation tolerance and preservation of seed longevity.

2.3.4.1 Seed germination

Seeds depend on degradation of stored carbon reserves for energy during germination. These reserves are mostly in the form of starch, soluble sugars, oil and proteins. In addition, stored RFO may play an important and essential role in the early stages of germination (Downie and Bewley 2000; Blöchl et al 2007) when the breakdown of other stored reserves are unable to meet the energy demand (Bewley and Black 1994). A reduction in RFO concentration upon germination is seen in legume seeds such as cow pea, jack bean, and soybean (Martin-Cabrejas et al 2008), where RFO degradation is almost complete before radicle protrusion (Horbowicz et al 1998; Lin et al 1998; Modi et al 2000). A role for RFO in germination is also supported by studies where inhibition of α-galactosidase activity by 1-deoxygalactonojirimycin (DGJ) significantly delays seed germination (Blöchl et al 2007). This inhibitory effect of DGJ is reversed by galactose application or partially by sucrose suggesting that galactose plays an important role in the sugar signalling pathway (Blöchl et al 2007). In contrast to previous reports, Dierking and Bilyeu (2009) recently reported that water imbibition and germination rate of soybean seeds with normal or low RFO concentrations is not significantly different. Nor does a difference in raffinose and stachyose concentrations affect germination rate and emergence time in soybean seeds (Neus et al 2005). Similarly, maize seed germination is not affected by the absence of raffinose (Brenac et al 1997). These studies suggest that utilization of RFO in the initial germination processes may not be absolutely required for germination.

2.3.4.2 Seed desiccation

Desiccation-tolerant seeds survive conditions wherein most of their cellular water is removed. The process of desiccation tolerance is attributed to the accumulation of soluble sugars (Blackman et al 1991) and certain late embryogenesis proteins (Bartels et al 1988). RFO accumulation during seed maturation has led to the speculation that they have an important role as osmoprotectants and are needed to enhance seed storage longevity (Horbowicz and Obendorf 1994; Obendorf 1997). This seems to be true for maize seeds as desiccation tolerance is not observed in the absence of raffinose (Brenac et al 1997). The role of RFO in desiccation

tolerance may be to protect plant cell membranes by the formation of glassy state (Burke 1986). In this process, the viscosity and cohesive forces among molecules in cytoplasm of drying seeds increases with water loss, resulting in reduced mobility and glass formation. The roles of glasses include the maintenance of structural and functional integrity of macromolecules (Slade and Levine 1994; Roose 1995), prevention of conformational changes in proteins (Prestrelski et al 1993) and stabilization of enzymes (Chang et al 1996). The hydroxyl groups of RFO are known to replace water around polar residues in membrane phospholipids, thus stabilizing and maintaining the integrity of cellular membranes and proteins during desiccation (Bryant and Wolfe 1992; Vertucci and Farrant 1995; Crowe et al 1998).

2.3.4.3 Cold acclimation and low-temperature tolerance

Cold acclimation in plants leads to an accumulation of RFO and several studies have suggested a role for RFO in freezing tolerance (Bachmann et al 1994; Castonguay et al 1995; Castonguay and Nadeau 1998; Gilmour et al 2000). However, some studies have contradicted the role of oligosaccharides in cold acclimation, drought tolerance and in seed germination. The freezing tolerance of non-acclimated leaves and their ability to cold acclimate is not affected in an *Arabidopsis thaliana* mutant lacking raffinose synthase gene and raffinose. This suggests that raffinose is not required for freezing tolerance or for cold acclimation in *Arabidopsis thaliana* (Zuther et al 2004). A similar observation was made nn perennial rye grass, where drought stress neither increased the concentrations of loliose and RFO nor the activities of loliose synthase (tentatively termed) and raffinose synthase (EC 2.4.1.82). Loliose [α-D-Gal (1, 3) α -D-Glc (1, 2) α -D-Fru] is a trisaccharide structurally similar to raffinose, however, its role and biosynthetic pathway is unknown. The concentrations of raffinose precursors, myo-inositol and galactinol, as well as the gene expression of myo-inositol 1-phosphate synthase (EC 5.5.1.4) and galactinol synthase (EC 2.4.1.123) are unaffected by drought stress in perennial ryegrass (Amiard et al 2003). These results also suggest that these sucrosyl-galactosides might not have any major role in drought tolerance. The functional roles of sugars in cellular stress tolerance (Tunnacliffe and Lapinski 2003) and germination of seeds (Dierking and Bilyeu 2009) have also been recently challenged.

2.3.4.4 RFO as free radical scavengers

Various abiotic stresses produce excess concentrations of reactive oxygen species (ROS). The generation of ROS such as the superoxide radical anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$) beyond the antioxidant capacity of a biological system causes oxidative stress (Aruoma et al 1997; Shigeoka et al 2002). Soluble carbohydrates like RFO and sugar alcohols act as antioxidants by scavenging hydroxyl radicals and protecting plants from oxidative damage (Nishizawa et al 2008a). Transgenic *Arabidopsis thaliana* plants overexpressing the heat shock transcription factor A2, produce higher levels of galactinol and raffinose compared with wild-type plants under control growth conditions and have increased tolerance to methylviologen treatment, salinity and chilling stress (Nishizawa et al 2006). Similarly, galactinol and raffinose can effectively protect salicylate from attack by hydroxyl radicals (indicated by the suppression of formation of 2,3-dihydroxy-benzoic acid *in vitro*) showing the role of RFO as antioxidants in *Arabidopsis thaliana* leaves (Nishizawa et al 2006; 2008b). The reaction between $^{\bullet}OH$ and a hydroxylated compound like a sugar involves abstraction by $^{\bullet}OH$ of a weakly bonded hydrogen atom from the substrate with production of water and a free radical as follows (Walling 1957; Green 1980).

$$(\text{H-C-OH})\,n + {}^{\bullet}\text{OH} \rightarrow ({}^{\bullet}\text{C-OH})n + \text{H2O}$$

The quenching efficiency and stability of sugar has been linked to its ability to regenerate itself by abstracting a hydrogen atom from the medium resulting in higher stability and more efficient $^{\bullet}OH$ scavenging of sugar molecule (Morelli et al 2003). Among simple sugars, the highest antioxidant activities have been reported for sucrose and maltose, intermediate activities for glucose and fructose and low activities for sorbitol (Morelli et al 2003). The antioxidant activities of galactinol and raffinose are similar when assayed *in vitro* (Nishizawa et al 2008b). The oxidized RFO radicals produced as a result of reaction with $^{\bullet}OH$ may be regenerated by ascorbic acid or other reducing antioxidants, such as flavonoids (Agati et al 2007). Results of these studies, therefore, indicate that RFO play an important role in plants responses to environmental stress conditions and that removal of RFO by modification of RFO biosynthetic pathway may negatively affect plants ability to perform or survive under stressful environmental conditions.

2.4 RFO structure and biosynthesis

2.4.1 RFO structure

RFO are soluble carbohydrates which contain linear galactosyl residues attached to the glucose moiety of sucrose via a α-(1→6) glycosidic linkage (Avigad and Dey 1997; Fig 2.4). These non-reducing sugars can be regarded as derivatives of sucrose with a varying number of galactosyl residues attached (Dey 1980). The most common forms of RFO include raffinose, stachyose, verbascose and ajugose which are tri-, tetra-, penta-, and hexasaccharides, respectively (Fig. 2.4). Dicotyledonous plants primarily accumulate stachyose and verbascose, whereas raffinose is the major RFO in monocotyledonous plants (Peterbauer and Richter 2001).

2.4.2 Biosynthesis of RFO in plant cells

2.4.2.1 RFO synthesis in cell cytoplasm

RFO biosynthesis in cell cytoplasm starts with the formation of raffinose by reversible addition of galactose units from galactinol to sucrose by raffinose synthase (Lehle and Tanner 1973; Obendorf et al 2009; Fig 2.5). Further chain elongation is achieved by addition of galactosyl residues from galactinol by stachyose and verbascose synthases to form next higher degree of polymerized RFO, stachyose and verbascose, respectively (Tanner and Kandler 1968; Obendorf et al 2009; Fig. 2.5). This form of RFO biosynthesis is designated as galactinol dependent pathway.

2.4.2.2 RFO synthesis in the vacuoles

Biosynthesis of long chian RFO in vacuoles is catalyzed by galactan-galactan galactosyl transferase (GGT) independently of galactinol (Bachmann et al 1994; Bachmann and Keller 1995; Obendorf et al 2009; Fig 2.5). GGT, a glycoprotein, transfers the terminal galactosyl residue of a stachyose to a second stachyose molecule producing verbascose and raffinose (Braun and Keller 2000; Bachmann et al 1994). The synthesis of RFO in vacuoles requires transport of stachyose from cell cytoplasm into vacuols through tonoplast. The stachyose concentration is higher in cytosol compared to vacuoles; thus the transport of stachyose might occur against a concentration gradient. The transport of stachyose in the vacuoles may be stimulated by MgATP and PPi and operated by an H^+-sugar antiport system similar to uptake of stachyose in Japanese Artichoke (Keller 1992; Greutert and Keller 1993).

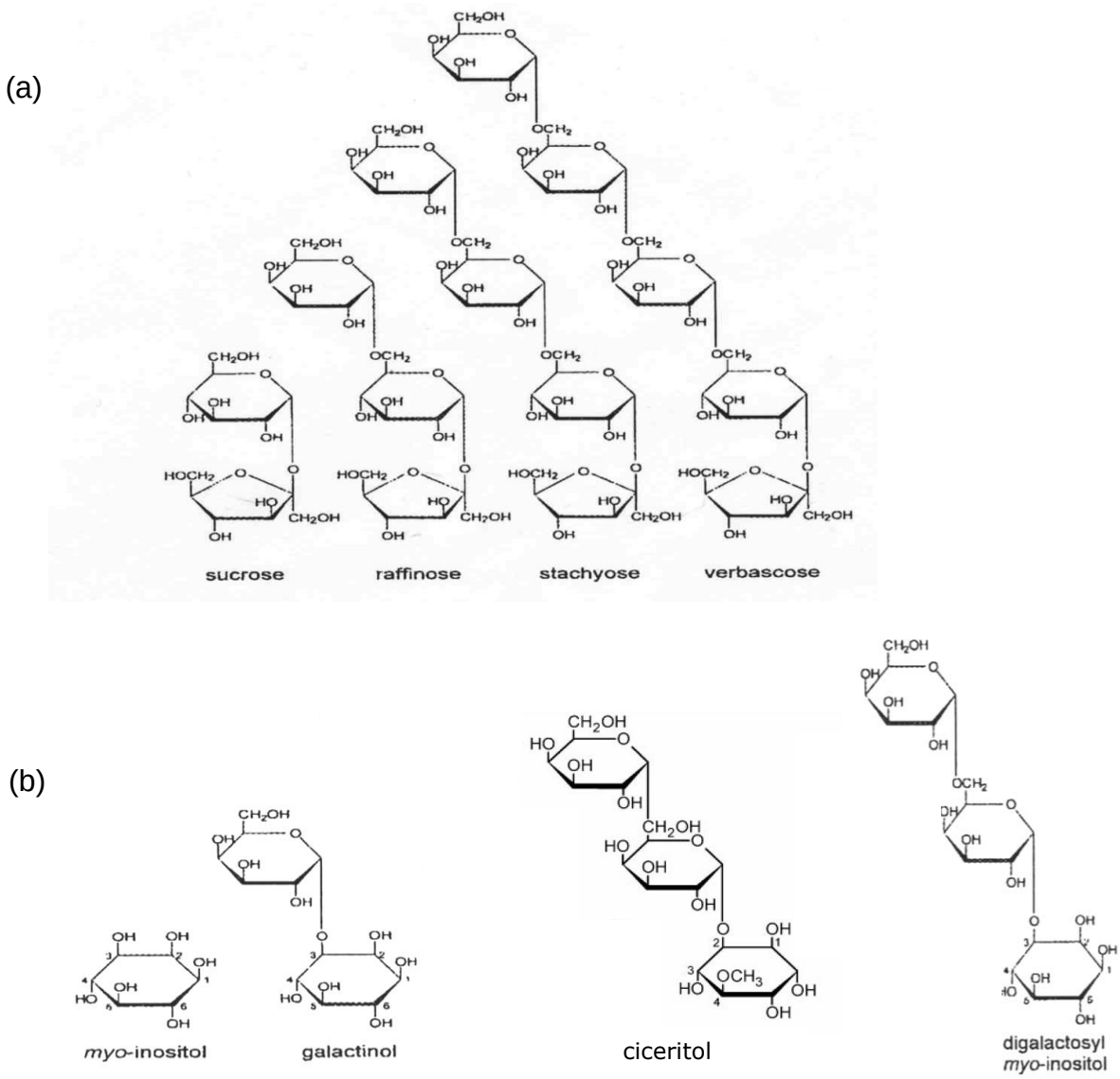

Figure 2.4. Structures of (a) sucrose and major RFO and (b) cyclitols in plants. (Obendorf 1997; Horbowicz et al 1998).

2.4.2.3 RFO synthesis in different tissues and transport

Studies on RFO synthesis in common bugle (*Ajuga reptans* L.) leaves have shown that RFO biosynthesis is compartmentalized to two plant tissues (Fig 2.5). A storage pool of RFO is synthesized in mesophyll cells, whereas a transport pool is produced in phloem intermediary cells (companion cells) (Bachmann et al 1994; Bachmann and Keller 1995). Stachyose is transported to long-distances in many plants and functions as a source of energy and carbon reserves (Bachmann and Keller 1995). The synthesis of stachyose to be transported, starts with diffusion of sucrose synthesized in mesophyll through plasmodesmata into intermediary cells of phloem, where transport stachyose is synthesized by galactinol-dependent pathway (Bachmann and Keller 1995; Fig 2.5). The large size of stachyose hinders it's diffusion into mesophyll and leads to the build up of a concentration gradient in intermediary cells that drives stachyose to enter the phloem sieve elements (Turgeon and Gowan 1990; Turgeon 1991).

2.4.2.4 Galactosyl cyclitol synthesis

Galactosyl cyclitols (cyclitol α-galactosides) are synthesized in cell cytosol and chloroplast and have the same role as RFO in seeds (Horbowicz and Obendorf 1994; Horbowicz et al 1998). Similar to major RFO, galactosyl cyclitols are formed by transfer of galactosyl residues from a galactosyl donor to an acceptor molecule e.g. D-pinitol. The transfer of a galactosyl residue from galactinol to D-pinitol by stachyose synthase forms monogalactosides: galactopinitol A and galactopinitol B (Obendorf et al 2009). A subsequent transfer of galactosyl residue to galactopinitol A or B forms digalactoside ciceritol. Similarly, a galactosyl residue transfer from UDP-Gal to D-chiro-inositol starts the biosynthesis of fagopyritol B1, which is a major α-galactoside in buckwheat seed embryos (Obendorf et al 2000).

2.4.2.5 The role of galactinol synthase in RFO biosynthesis

Galactinol synthase (EC 2.4.1.123) catalyzes the formation of galactinol from UDP-galactose and myo-inositol in plants (Loewus and Murthy 2000). The enzyme is localized in the cytoplasm together with raffinose and stachyose synthase (Bachman and Keller 1995; Peterbauer et al 2002b).

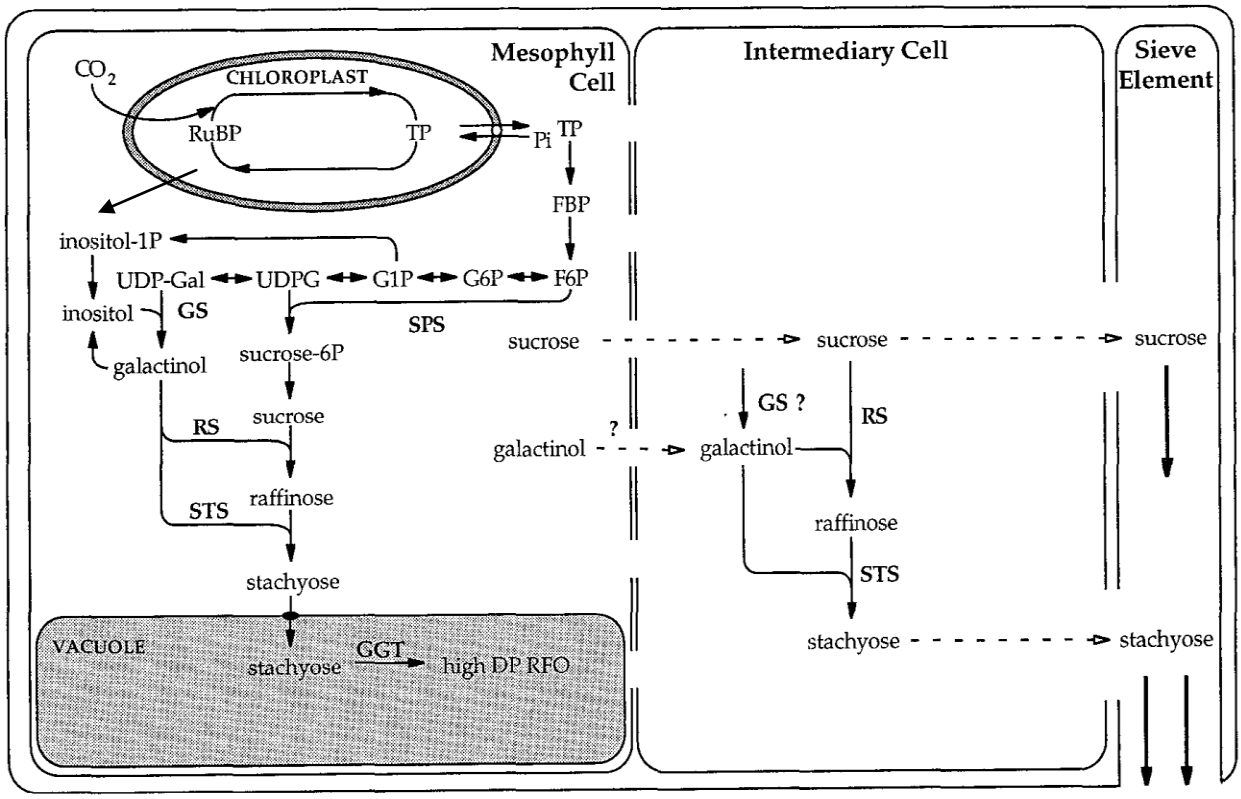

Figure 2.5. Inter- and intracellular compartmentation of RFO biosynthesis in leaves of *Ajuga reptans*.

Abbrevations: GS (galactinol synthase), RS (raffinose synthase), STS (stachyose synthase), SPS (sucrose phosphate synthase), DP (degree polymer) UDP-Gal (UDP- galactose), RuBP (ribulose-1, 5-bisphosphate), TP (triose phosphate), G6P (glucose 6-phosphate), F6P (fructose 6-phosphate), FBP (fructose bi phosphate), G1P (glucose 1-phosphate), UDPG (UDP-glucose) (Bachmann and Keller 1995).

Galactinol synthase activity was first detected in crude extract of maturing pea seeds (Frydman and Neufeld, 1963) and since then its activity has been reported in seeds and vegetative parts of several plant species (Keller 1992a; Liu et al 1995; Ribeiro et al 2000; Taji et al 2002). Galactinol synthase is a monomeric 38 - 43 kDa polypeptide, which requires Mn^{2+} for enzymatic activity and has a pH optimum between 5.6 and 7.5 depending on Mn^{2+} concentration. A putative manganese-binding motif, DXD is conserved in most galactosyl transferases and is present in all galactinol synthases (Busch et al 1998). The enzyme activity is optimal at approximately pH 7.5, but dramatically decreases when pH is increased to 8.0. A second pH optimum, but with lower activity, exists at pH 5.5 (Sprenger and Keller 2000).

Different isoforms of galactinol synthases have been isolated from plant species. *Ajuga reptans* galactinol synthases known as GolS-1 and GolS-2 are induced by cold. The GolS-1 form is considered to be responsible for the biosynthesis of storage RFO in mesophyll cells, whereas the GolS-2 form has a role in the biosynthesis of transport RFO in companion cells. The activity of GolS-1 is higher as compared to GolS-2 form (Sprenger and Keller 2000). The cDNA nucleotide and protein amino acid sequences of the two galactinol synthases from *Ajuga reptans* are 70% similar, and carry a serine phosphorylation site and hydrophobic pentapeptide at the carboxy terminal (Sprenger and Keller 2000).

Seven galactinol synthase genes have been identified in *Arabidopsis thaliana* (Taji et al 2002) and three of these (AtGolS1, 2 and 3) are believed to be stress responsive. AtGolS1 and 2 are induced by drought and high-salinity stresses and AtGolS3 is induced by cold. Heat shock transcription factors (HSF) regulate the expression of *Arabidopsis thaliana* GolS1 and GolS2 (Panikulangara et al 2004; Busch et al 2005; Schramm et al 2006). Transgenic *Arabidopsis thaliana* plants overexpressing GolS2 show increased levels of endogenous galactinol and raffinose under non-stress conditions. These studies suggest that stress-inducible galactinol synthase may play a key role in the accumulation of galactinol and raffinose under drought conditions, when the molecules may function as osmoprotectants (Taji et al 2002).

Galactinol synthase homologues isolated from buckwheat (*Fagopyrum esculentum* Moench) seed catalyze the synthesis of fagopyritol A1, fagopyritol B1 and galactinol. These homologues, designated FeGolS-1 and FeGolS-2, show galactinol synthase activities in the presence of UDP-galactose and myo-inositol. In the presence of UDP-galactose and D-chiro-inositol, FeGolS-1 catalyzes the synthesis of fagopyritol B1 whereas FeGolS-2 catalyzes the

synthesis of both fagopyritol A1 and fagopyritol B1. FeGolS-1 is closely related to soybean galactinol synthase, whereas FeGolS-2 belongs to a distinct galactinol synthase group which is closely related to rice (*Oryza sativa L.*) galactinol synthase (Ueda et al 2005). Galactinol synthase may have a key regulatory role in carbon partitioning between sucrose and RFO (Saravitz et al 1987).

2.4.2.6 Myo-inositol biosynthesis (MI)

Recent studies show that myo-inositol and its synthesizing enzyme myo-inositol 1-phosphate synthase (MIPS) play an equally important role in RFO biosynthesis (Karner et al 2004). Myo-inositol or inositol is synthesized in two enzymatic steps. In the first step, D-glucose-6-phosphate is irreversibly cyclized to 1-L-*myo*-inositol 1-phosphate (1D-myo-inositol 3-phosphate) by MIPS (EC 5.5.1.4). In the second step, phosphate is removed by inositol phosphatase (EC 3.1.3.25), releasing free *myo*-inositol. Cytosolic and chloroplastic forms of MIPS have been characterized (Chaudhuri et al 1997) and found to be homo-trimeric (cytosolic form) and homo-tetrameric (plastidial form). The biochemical and kinetic parameters of these two forms do not differ significantly from each other or to other cytosolic MIPS previously described (Majumder et al 1997). To study the effects of myo-inositol concentration on RFO accumulation, Karner et al (2004) fed exogenous myo-inositol to pea seeds which resulted in significant increase in galactinol, raffinose, and sucrose, confirming the significant impact of myo-inositol on RFO accumulation. A single point mutation in soybean MIPS reducing MIPS enzymatic activity decreases seed myo-inositol and RFO concentration (Hitz et al 2002). Reduction of myo-inositol concentration by antisense suppression of MIPS activity leads to lower galactinol and raffinose content in potato (*Solanum tuberosum* L.) leaves, confirming MIPS important role in RFO biosynthesis (Keller et al 1998).

2.4.2.7 Raffinose and raffinose synthases (RFS) in plants

Raffinose synthase is considered the key enzyme that channels sucrose into RFO biosynthetic pathway. The enzyme (RFS) (EC2.4.1.82) catalyzes the transfer of galactosyl residue from galactinol [*O*-α-D-galactopyranosyl-(1→1)-*myo*-inositol] to sucrose to form trisaccharide raffinose, the first member of RFO.

Reaction:

RFS

Galactinol + Sucrose → myo-inositol + Raffinose

RFS have been purified from fababean (*Vicia faba* L.*)* (Lehle and Tanner 1973) and common bugle (*Ajuga reptans* L.) leaves (Bachman et al 1994). RFS exhibits optimum catalytic activity at pH 7.0 and at 45 °C (Lehle and Tanner 1973; Peterbauer et al 2002a; Li et al 2007). RFS uses galactinol, galactosyl ononitol (a methylated derivative of galactinol) and p-nitrophenyl a-D-galactopyranoside as donors of galactosyl residues for raffinose synthesis and uses sucrose, D-ononitol, D-pinitol as acceptors. Higher RFO can not be synthesized by RFS; however, RFS can catalyze an exchange of galactinol between raffinose and sucrose (Lehle and Tanner 1973). Beta-galactose containing other disaccharides such as lactose, 4−β-galactobiose, *N*-acetyl-D-lactosamine, and lacto-*N*-biose can be used as acceptors by RFS isolated from rice (Li et al 2007). RFS from pea and fababean can also utilize p-nitrophenyl α-D-galactopyranoside, which is an artificial substrate with high affinity towards α-galactosidases, as a galactosyl donor. RFS biosynthetic activity is inhibited by 1-deoxygalactonojirimycin, and the hydrolytic activity is inhibited by sucrose (Peterbauer et al 2002b). Rice RFS of 85 kDa has a specific hydrolytic activity of 6.8 nkat mg^{-1}, 8.5 nkat mg^{-1}, and 5.7 nkat mg^{-1} toward *p*NP-α-Gal, galactinol, and raffinose, respectively (Li et al 2007).

RFS share some common conserved sequences common to α-galactosidase family 36 and 27, and share structural similarities at the active site (Lehle and Tanner 1973; Peterbauer et al 2002a). Structural and biochemical similarities are also found to seed imbibition proteins and stachyose synthases (Peterbauer et al 2002a). Pea RFS has a molecular mass of 88.7 kDa and contains a conserved aspartic acid residue, which acts as a catalytic nucleophile to generate a covalent glycosyl-enzyme intermediate similar to α-galactosidases of family 27 (Peterbauer et al 2002b).

2.4.2.8 Stachyose biosynthesis in plants

Stachyose is the main soluble carbohydrate in seeds of a number of important crops. Stachyose and myo-inositol are co-produced when a galactosyl residue from galactinol (*O*-a-D-galactopyranosyl-*myo*-inositol) is transferred to raffinose by the action of stachyose synthase

(STS; EC 2.4.1.67) (Tanner and Kandler 1968; Holthaus and Schmitz 1991), and it contains extra 80 amino acids, when compared to RFS.

Galactopinitol A and galactosyl ononitol can replace galactinol as galactose donor to form stachyose from raffinose. Galactosyl ononitol-dependent synthesis of stachyose has been demonstrated for adzuki bean seed (Peterbauer and Richter 1998; Peterbauer et al 1999). The enzyme catalyzing this reaction performs a double displacement reaction as follows (Peterbauer et al 2002b):

$$\text{Galactinol} + E \rightarrow \text{myo-inositol} + (E\text{-Gal})$$

$$(E\text{-Gal}) + \text{raffinose} \rightarrow E + \text{stachyose}$$

A glycosyl - enzyme intermediate is formed first and the enzyme releases the first product myo-inositol before forming a complex with an acceptor. In addition to stachyose biosynthesis in plants, STS purified from adzuki bean can form galactosyl ononitol (*O*-a-D-galactopyranosyl-(1 3 3)-4- *O*-methyl-D-*myo*-inositol) from galactinol and D-ononitol (1D-4-*O*-methyl-*myo*-inositol (Peterbauer et al 1998; Peterbauer and Richter 1998). Adzuki bean (*Vigna angularis* Willd. Ohwi and Ohashi) seeds galactosyl ononitol is considered a methylated analogue of galactinol (Richter et al 1997) and it can serve as galactosyl donor to raffinose. In lentil, soybean, and chickpea galactopinitol A (*O*-a-D-galactopyranosyl- (1 3 2)-4-*O*-methyl-D-*chiro*-inositol), ciceritol (*O*-a-D- galactopyranosyl-(1 3 6)-*O*-a-D-galactopyranosyl-(1 3 2)-4-*O*-methyl-D-*chiro*-inositol), galactopinitol B (*O*-a- D-galactopyranosyl-(1 3 2)-3-*O*-methyl-D-*chiro*-inositol) and fagopyritol B1 (*O*-a-D-galactopyranosyl-(1 3 2)-D-*chiro*-inositol) are the major galactosyl cyclitols (Fig 2.5). It has been shown that STS catalyze the synthesis of these compounds (Hoch et al 1999). A summary of these reactions is given below:

Lentil STS

$$\text{Galactinol} + \text{D-pinitol} \rightarrow \text{Galactopinitol B}$$

$$\text{Galactinol} + \text{D-chiro-inositol} \rightarrow \text{Fagopyritol B1}$$

Adzuki STS

$$\text{Galactinol} + \text{D-ononitol} \rightarrow \text{Galactosyl ononitol}$$

Purified STS from various seeds i.e. kidney bean, adzuki bean and lentil (Tanner and Kandler 1968; Peterbauer and Richter 1998; Hoch et al 1999) exhibits physicochemical properties similar to those of RFS, like pH optima between pH 6.5 and 7.0. Variations among STS of different plant species have been noticed with regard to molecular weights, which range

from 85 to 95 kDa. STS of lentils is a 88.6 kDa monomeric protein with pI of 4.7 (Hoch et al 1999). The optimal temperature for STS activity is around 35 °C with one-half-maximum activity at approximately 23 °C.

The multifunctional STS from pea seed is a 95 kDa protein which has 83% similarity in amino acid sequence to the STS from adzuki bean, 46 to 56% similarity to the RFS and 28 to 30% similarity to a seed imbibition protein. The enzyme has broad acceptor specificity and synthesizes verbascose and ajugose besides self-transfer of stachyose to another stachyose molecule (Peterbauer et al 2002b). The donor of galactosyl residue for biosynthesis of verbascose and aujogose is stachyose or galactinol. These two substances compete for the active site of STS; however, galactinol is preferred. Thus, the synthesis of verbascose by STS through the galactinol-independent pathway *in vivo* proceeds when the ratio of stachyose to galactinol is very high. Incubation of pea seed STS with galactinol and verbascose, results in production of a low amount of ajugose (Peterbauer et al 2002b). STS can also hydrolyze the terminal galactose residue of its substrate; however, the hydrolytic activities are lower compared to the rate of transfer reaction from galactinol to raffinose. STS cannot start the biosynthesis of raffinose family oligosaccharides when incubated with galactinol and sucrose (Peterbauer et al 2002b).

2.4.2.9 Verbascose biosynthesis in plants

Verbascose is found in storage organs of many plant species and particularly in seeds of legumes (Dey 1985). Very little is known about the functions of verbascose in seeds, but it is assumed that like all other RFO members, it plays a role in desiccation tolerance and act as source of carbon reserve in seeds (Obendorf 1997). Compared to RFS and STS, very little is known about verbascose synthesizing enzyme. Previously, verbascose synthase (VRS) activity from fababean seeds was reported to co-purify with STS activity (Tanner et al 1967). More recently, it is suggested that a multifunctional STS is responsible for the biosynthesis of verbascose (Peterbauer et al 2002b) as follows:

$$\text{E-Gal} + \text{stachyose} \rightarrow \text{E} + \text{verbascose}$$

$$\text{Stachyose} + \text{stachyose} \rightarrow \text{raffinose} + \text{verbascose}$$

Mutations in the multifunctional STS are associated with lower ability to synthesize verbascose in pea (Peterbauer et al 2002b). A pea genotype with reduced verbascose concentration showed a total of 23 base pair variations within the STS transcript. These

variations resulted in 12 amino acids substitutions in STS and the enzyme produced verbascose when incubated with galactinol and stachyose or stachyose alone. These mutations, some of which are located in a block of 80 amino acids residues, have greater effect on the enzyme's ability to utilize stachyose as substrate than raffinose, using galactinol as galactosyl donor (Peterbauer et al 2002b).

2.4.2.10 Soybean lines with low RFO concentration

Soybean (*Glycine max* L.) mutant lines with a mutation in MIPS produce reduced raffinose and stachyose levels in seeds (Sebastian et al 2000). These mutant lines also contain less phytic acid than wild genotypes. The MIPS mutations reduce the enzyme activity and consequently myo-inositol concentration, which affects biosynthesis of galactinol, RFO and phytic acid (Hitz et al 2002). These MIPS mutant lines have been used to develop of cultivars for soybean meal production; however, their yield is lower than conventional soybean cultivars (Skoneczka et al 2009). Mutations in the RFO biosynthetic pathway in other legume crops have not been reported as yet. Mutation in RFS and STS may similarly affect RFO concentration and are potential targets for modification of RFO concentration in plants.

In conclusion, though more research work has been done in the last 10-15 years to understand RFO biosynthesis in plants, there are still large gaps in our knowledge of RFO pathways and RFO physiological role in plants. Elucidation of the roles of galactinol synthase and its different isoforms, mechanism of regulation of RFO biosynthesis, relationship between RFO and galactosyl cyclitols biosynthesis through common substrates/enzymes are critical for understanding RFO biogenesis and metabolism in plants.

2.5 Environmental effects on lentil growth, development and yield

2.5.1 Genotype by environment (G × E) interaction effects, types and lentil adaptation

Lentil yield and production is affected by several abiotic and biotic stresses in combination with crop management conditions. Drought during seed filling stages is known to reduce lentil yield (Summerfield et al 1989). High temperatures, which are often associated with drought may also negatively affect symbiosis with Rhizobia followed by lower nitrogen fixation and yields (Malhotra and Saxena 1993). Small-seeded lentil genotypes generally cope better with dry environmental conditions than large-seeded varieties due to their shorter seed filling period

(Erskine 1996). Varieties with early vigour and pod set generally produce higher yields compared to later-maturing varieties by escaping drought (Leport et al 2003). Therefore, development and selection of lentil genotypes with early seedling establishment, early flowering and maturity and rapid biomass development have been used to lessen drought stress and associated yield losses.

A major objective in lentil breeding is to develop cultivars that are high yielding and are stable over different environments. Crop yield and trait stability; however, are influenced by the genotype interactions with environmental (G × E) conditions. G × E interaction effects appear when genotypes/cultivars perform differently in different environments such that one genotype may have the highest performance in one environment but performs poorly in other environments (Acquaah 2007). The environmental factors may be (i) abiotic such as drought, heat, cold, salinity, mineral toxicity and deficiency, oxidation and water logging (ii) biotic such as pests and diseases or (iii) management conditions. Together these, factors produce G × E interactions by affecting various physiological and biochemical processes, plant growth and development (Basford and Cooper 1998). In the presence of G × E interaction effects, the differences between genotypes may vary widely among environments (DeLacy et al 1990; Annicchiarico 1997). The three basic types of G × E interaction effects are no interaction, non-crossover interaction and crossover interactions (Acquaah 2007). Multi-environment and regional trials are, therefore, used to assess the performance of genotypes and to evaluate and better understand the type, size and reasons of G × E interactions. A proper experimental design and statistical analysis are required to detect the presence of G × E interactions effects. The presence of a significant effect e.g. genotype × soil type may call for the development of a cultivar for a particular agro-ecological zone or the selection of a cultivar with superior performance.

The effects of genotypic, environmental and G × E interactions on agronomic as well as some major quality traits have been investigated over the years to understand lentil adaptation and responses to environmental conditions (Table 2.5). The presence and nature of these interactions, therefore, must be investigated to develop cultivars with higher yields or improved quality characteristics. Long days and warm temperatures are known to expedite flowering in lentils (Summerfield et al 1985) with different genotypes having different sensitivities to

temperature and photoperiod. Lentil seed shape and size are important physical characteristics due to their influence on seed processing operations. Seed diameter, thickness and shape of the edges (rounded or sharp) affect the de-hulling or splitting efficiency. Overall, round seeds are easier to decorticate than flat seeds. Studies conducted to find the effect of genotype and environment on lentil seed shape showed a strong genotypic control of seed shape (Williams et al 1993; Iliadis 2003).

Cooking quality is an important quality characteristic in lentils. It is defined as the time needed from the start of boiling until the seeds become soft (Iliadis 2003). Lentils with short cooking times are strongly preferred by consumers. Significant genotype by location interaction effects for cooking (texture) were reported for two lentil cultivars grown at 12 locations (Bhatty 1983). Genotype had a small effect on the texture of cooked lentils when compared to location. Available soil phosphorus levels and its effects on phytic acid concentration in the seed lead to these G × E effects on cooked lentil texture (Bhatty 1984). Reduction of cooking time with increased levels of seed phytic acid and soil phosphorus was later confirmed by Bhatty (1995). No significant G × E interaction effects for cooking time or seed phosphorus concentration or seed weight were observed for 24 lentil genotypes grown at three environments in Syria and Lebanon (Erskine et al 1985).

Genotype, environment and their interaction effects affect most lentil seed constituents. Wang and Daun (2006), using protein concentration as an indicator of environmental condition, investigated the effect of genotype and environment on seed constituents of four lentil genotypes grown in western Canada. The starch concentration was found to be significantly effected by genotype and the environmental conditions whereas the acid detergent fiber and neutral detergent fiber concentration was only affected by genotype (Wang and Daun 2006). Among soluble sugars, stachyose and sucrose were significantly affected by genotype; however, neither genotype nor environment affected raffinose and verbascose concentration (Wang and Daun 2006). A significant genotype / genotype × environment effect was also observed for fat and phytic acid concentration of lentil genotypes. Similarly, significant genotypic and environmental effect on several mineral constituents has been recently reported (Thavarajah et al 2009; 2011; Table 2.5).

2.5.2 Effects of growing conditions on RFO concentration and composition in plants

Effects of environmental conditions on protein, starch, fats, ADF, NDF, mineral as well as other traits in pulses and other crops have been studied (Reichert and MacKenzie 1982; Yang et al 1999; Kumar et al 2003, 2005; Wang et al 2006). Abiotic stresses affect plant physiological processes and have been shown to affect soluble carbohydrates concentrations in plants. However, relatively few studies have so far been conducted on the influence of environmental conditions and on G × E interactions effects on RFO content and composition.

2.5.2.1 Temperature

Raffinose, sucrose, and proline accumulate in response to low temperature stress in *Arabidopsis thaliana* plants (Nanjo et al 1999, Gilmour et al 2000, Taji et al 2002). Significantly higher soluble carbohydrates and a three-fold difference in stachyose content is seen in buckwheat *(Fagopyrum esculentum* Moench) seeds developing at lower temperature (18 °C) compared to seeds developing at higher temperature (25 °C) (Horbowicz et al 1998). Similarly, soluble carbohydrates content in yellow lupin seeds varies at different growing temperature regimes (Piotrowicz-Cieslak 2006). Yellow lupin seeds that mature at constant temperature (26 °C for 24 hr) accumulate more raffinose than seeds that mature at an optimum temperature regime (24 °C for 12 hr and 19 °C for the next 12 hr). Seeds maturing at high temperature (26 °C for 24 hr) accumulate less stachyose and verbascose than those maturing at optimum temperature conditions. In seeds maturing at high - low temperature (26 °C for 16 hr and 4 °C), raffinose decreases whereas the level of stachyose and verbascose increases, compared to those maturing at optimum conditions. Similarly, sucrose, D-galactose and myo-inositol contents in seeds maturing at optimum temperatures is lower than in seeds maturing at both high and high-low temperature regimes (Piotrowicz-Cieslak 2006). These findings suggest that different environmental conditions not only affect RFO concentration but also composition of individual RFO in seeds. However, contrary to the finding of Piotrowicz-Cieslak (2006), Górecki et al (1996) reported only minor differences in RFO contents in white lupin seeds that matured at different temperature. More recently, Hao et al (2010) reported that the ratio of raffinose to total carbohydrates increased whereas the ratio of sucrose to total carbohydrates declined in melon leaves when produced at low night temperature (9 °C) as compared to the normal (15 °C) night

temperature. Thus, the data suggested an effect of temperature on carbon partitioning from sucrose to raffinose-family oligosaccharides (RFO).

2.5.2.2 Water

Soil drought affects soluble carbohydrates concentration in seeds. Among soluble carbohydrates sucrose plays an important role in plant adjustment to drought stress (Keller and Ludlow 1993). Other soluble carbohydrates such as cyclitols accumulate during soil drought and play an important role in plants osmotic adjustment (Wanek and Richter 1997). Accumulation of cyclitols in vacuoles reduces water potential of cells facilitating water uptake from the environment (Orthen et al 1994). Similarly, RFO accumulate in vegetative tissues in response to drought stress (Taji et al 2002). Piotrowicz-Cieslak et al (2006) reported a two-fold increase in soluble carbohydrates and RFO concentration in two lupin species grown under water deficit conditions. Relatively few studies, however, have investigated the effects of different environmental conditions on RFO content and composition under field conditions on crop plants in general and lentil in particular. A study by Wang and Daun (2006) revealed very little environmental effects on sugar content of lentil cultivars grown in western Canada; however, the authors did not provide any information on the differences in environmental conditions. More recently, Kumar et al (2010) reported a significant genotypic and G × E effect on RFO and sucrose concentration of various soybean cultivars grown at different geographical locations in Indore (22° N), Palampur (32° N), and Bangalore (12° N) with mean max-min temperature of 31.6 - 23.8 °C, 25.4 - 14.8 °C and 25.5 - 19.8 °C, respectively.

2.5.3 Heritability

Heritability is the proportion of observed variation in a progeny that is inherited or the ratio of genetically caused variation to total variation (Acquaah 2007). The phenotypic variance (V_P) of a trait can be expressed as follows:

$$V_P = V_G + V_E + V_{GE}$$

V_G, V_E and V_{GE} indicate genetic variance, environmental variance and interaction between genetic and environmental variance, respectively. Genetic variance is composed of additive genetic variance (V_A), dominance variance (V_D) and the nonallelic or epistasis variance (V_I) and can be written as follows:

$$V_G = V_A + V_D + V_I$$

Table 2.5. Studies on genotype and environmental effects on agronomic and seed constituents in lentil.

Trait	Effect	Reference
Yield	G×E*, E*, G*,	Sarkar et al 2010; Dehghani et al 2008; McKenzie and Hill 2004; Solanki and Singh 2000
Time to flowering	G×E*	Erskine et al 1989
Seed size	G*, G×L^{ns}	Erskine et al 1985
Seed shape	G*, E ^{ns}	Williams et al 1993; Iliadis 2003
Dehulling/decortication	G*, E^{ns}	Erskine et al 1991
Seed weight and cooking time	G×L^{ns}	Erskine et al 1985
Cooking time (Texture, measured using sheer force)	G×L*, G×L*, G×Y*, L*,	Bhatty 1983; Bhatty 1984; Bhatty 1995
Starch concentration	G*, E*	Wang and Daun 2006
RFO and sucrose concentration, Raffinose, verbascose	G×E^{ns}, G^{ns}, E^{ns}	Wang and Daun 2006
ADF	G*	Wang and Daun 2006
Fat, potassium and zinc concentration	G*, G×E*	Wang and Daun 2006
Phytic acid concentration	G×E*	Wang and Daun 2006
Selenium concentration	G*, L*	Thavarajah et al 2011
Iron and zinc concentration	G*, L*, G×L*, G×Y*	Thavarajah et al 2009
Phytic acid, zinc and iron concentration	E* (Increasing temperatures)	Thavarajah et al 2011

G= Genotype, E= Environment, L= Location, Y= Year,

*= Significant, ns= Non significant

*Wang and Daun 2006, used protein content as a indicator of general environmental effect

The additive components of genetic variance are contributed by genes and produce linear additive effects. The resemblance between parents and offspring is largely due to additive genetic effects. The dominance effects on the expression of a quantitative character are generally small in comparison with additive effects. Epistatic effects are smaller than the additive and dominance effects and are ignored in calculating heritability. The two different estimates of heritability are the broad sense and the narrow sense heritability. Heritability estimated using the total genetic variance (V_G) is called the broad sense heritability because it estimates heritability on the basis of all genetic effects and is expressed in terms of variances as follows (Acquaah 2007):

$$h^2 = V_G/V_P$$

Where,

V_G= variance due to the genotype, V_P = phenotypic variance or total variance.

If additive genetic effect is used to determine heritability, then the estimate of heritability becomes narrow sense heritability which is more useful for plant breeders since additive genetic effect determines response to selection. It can be expressed as follows:

$$h^2 = V_A/V_P$$

For homozygous individuals broad sense heritability is equal to narrow sense heritability. Heritability estimates are grouped into three categories. Heritability estimates larger than 0.75 are considered high, estimates between 0.5 - 0.75 are considered moderate, whereas heritability estimates below 0.5 are considered low. The methods of estimating heritability are based on partitioning observed variation of a quantitative character into genetically and environmentally controlled components. The common methods for estimating heritability are variance component method using the analysis of variance and parent-offspring regression method. From analysis of variance heritability can be estimated as follows (Singh et al 1993; Schneiner and Lyman, 1989; Becker, 1984):

$$h^2 = \sigma^2_{GE}/ \sigma^2_P,$$

Where:

σ^2_{GE} = (MSGE - MSe) /r, (MSGE = genotype×environment mean square, e = number of environments, r = no of replicates).

$\sigma^2_P = \sigma^2_G + (\sigma^2_{GE}/e) + (\sigma^2 e/re)$, ($\sigma^2_G$ = (MSG-MSGE)/re and σ^2_{GE} = genotype×environment variance).

$\sigma^2 e$ = MSe, error mean square , σ^2_G = genotype variance and MSG = genotype mean square.

Heritability estimates are used to determine whether a trait can be improved through breeding. A high heritability estimate of a trait would indicate that the use of plant breeding methods will likely improve that trait. Another use of heritability estimate is to determine the most effective selection method. The heritability estimates of some lentil traits have been reported in literature. The heritability of lentil seed yield was reported to range from 0.14 - 0.95 (Sarker et al 2010). The broad sense heritability estimates of seed yield, average seed weight and cooking time in lentil were 0.50, 0.98 and 0.82, respectively (Erskine et al 1985). Similarly, the broad sense heritability estimates for protein, Fe and Zn concentrations in lentil seed were 0.71, 0.64 and 0.68, respectively (Erskine et al 1985; Thavarajah et al 2009). The heritability estimates of RFO concentration, however, have not been reported as yet.

2.6 Research hypotheses

High concentrations of RFO reduce lentil quality and consumption due to their anti-nutritional effects. Development of lentil cultivars with reduced RFO may significantly increase lentil consumption. A critical analysis of the published literature on RFO leads to the following hypotheses of my research:

1. Raffinose family oligosaccharides concentrations in lentils are affected by genotype and environmental conditions
2. Variation in RFO concentration and composition exists in lentil seeds
3. Galactinol synthase activity determines variation in RFO concentrations in lentil seeds

3 COMPOSITION AND ASSOCIATION BETWEEN MAJOR SEED CONSTITUENTS IN LENTIL GENOTYPES

3.1 Abstract

Development of lentil cultivars with increased seed amylose, protein and reduced concentration of anti-nutritional constituents are desired from lentil utilization and human health perspective. In selected lentil genotypes, we studied seed weight, seed coat weight and colour, seed composition and the association between these major quality traits. Significant ($P \leq 0.05$) variation existed for all traits except seed coat weight. The starch and protein concentration ranged from 39.4 - 45.3 g and 23.8 - 29.3 g 100 g^{-1}flour on dry weight basis whereas the amylose concentration ranged from 29.8 - 34.0 g 100 g^{-1}starch. Glucose, sucrose and RFO concentration of lentil genotypes ranged from 0.04 - 0.08 g, 0.7 - 2.4 g, and 4.6 - 6.6 mmoles 100 g^{-1}flour, respectively. Raffinose, stachyose and verbascose concentration varied from 1.6 - 2.4 g, 1.7 - 2.9 g, and 1.2 - 1.9 g 100 g^{-1}flour, respectively. A significant ($P \leq 0.05$) positive correlation existed between thousand seed weight and starch, thousand seed weight and RFO, and sucrose concentration. Similarly, a significant negative correlation ($P \leq 0$ .05) was found between starch and protein concentration, thousand seed weight and protein concentration, and thousand seed weight and amylose concentration. The lack of a significant correlation between RFO and other quality traits indicates that selection for low RFO concentration may not affect other important quality traits in lentil seeds.

3.2 Introduction

Lentil (*Lens culinaris* Medik. ssp. *culnaris*), a lens-shaped grain legume, that has been used extensively as a source of vegetable protein and it is an important component of the human diet in many parts of the world. The major lentil producing countries include Canada, India and Turkey. Canada is a leading lentil producing and exporting nation and 98% of lentils production is in Saskatchewan whereas the remaining 2% comes from Alberta. In 2009, Canada produced 1.5 million metric tonnes lentils compared to 1 million metric tonnes in 2008 (Statistics Canada,

http://www40.statcan.gc.ca/l01/cst01/Prim11b-eng.htm). The seed coat colour of lentils has been classified in to five classes: green, pink, brown, gray or black and the seed coats may also have patterns such as marbled, dotted, spotted or complex (or any combinations of four) (Erskine and Witcombe 1984; Vandenberg and Slinkard 1990). The cotyledons may be yellow, red or green. About half of the lentils grown in Canada have pale green seed coats and yellow cotyledons. The other half mostly has gray or brown seed coats and red cotyledons. Red cotyledon lentils are mostly consumed after removal of the seed coat by abrasive decortication (commonly referred to as dehulling) as intact or split cotyledons (dhal) or dhal flour, especially in South Asia and the Middle East. In Europe and parts of the Middle East, green lentils are mostly consumed as whole seed. Decorticated red lentils are normally used in soups, stews and salads. Decortication is an important process in lentil utilization and has been reported to improve palatability, taste and cooking time of several legumes including lentils (Singh and Singh 1992). It also reduces the tannin concentration which is mostly present in the seed coat (Wang et al 2008) and may reduce the associated antioxidant activities of tannins in lentil seeds.

Lentils, like all other pulses, are an excellent source of proteins, carbohydrates and fiber, and provide many vitamins and minerals (Chibbar et al 2010). Lentils contain relatively high amounts of lysine and provide a well balanced amino acid profile when consumed in combination with cereal based foods or foods that are rich in sulphur-containing amino acids (methionine and cysteine) and tryptophan (Boye et al 2010). Lentils are also a good source of dietary carbohydrates and energy. The carbohydrate fraction of lentils has attracted more attention recently for its health promoting as well as anti-nutritional properties (Chibbar et al 2010). The total carbohydrate concentration of lentil seeds varies from 43.3 - 74.9 g 100 g^{-1}dry matter (Ereifej and Haddad 2001; Cai et al 2002; El-Adawy et al 2003). Starch constitutes the major storage carbohydrate of lentil seeds and varies from 34.7 - 65.0 g 100 g^{-1}dry matter (Urbano et al 2007). Starch is composed of two glucan polymers amylose and amylopectin. Amylose consists of predominantly unbranched α-(1→4)-linked glucan chains whereas amylopectin is a highly branched molecule consisting of α-(1→4) and α-(1→6)-linked glucan chains. The proportions of amylose and amylopectin vary with plant species and provide their starches with unique functional and physicochemical characteristics (Blazek and Copeland 2008; Hoover et al 2010). The amylose concentration of lentil and other pulse starches vary from 20 - 45.5 g 100 g^{-1} (Urbano et al 2007; Chibbar et al 2010) compared to amylose concentration of

about 25 g 100 g^{-1} for cereal starches on dry weight basis (Ansari et al 2010). High amylose concentration in starch has been associated with reduced digestibility, low glycemic index and described as resistant starch (Sandhu and Lim 2008; Chibbar et al 2010). Foods containing resistant starches provide low glycemic index and are beneficial for the prevention of diseases such as diabetes, obesity, heart diseases and colon cancer and are considered an essential part of healthy diets that are increasingly desired for human consumption (Bjiirck and Asp 1994; Roberts 2000; Wolever and Mehling 2002; Brand-Miller et al 2003; Brand-Miller 2007; Chibbar et al 2010).

Lentil seeds contain high quantities of α-galactosides also known as raffinose family oligosaccharides (RFO). These water-soluble low molecular weight non-reducing sugars are sucrose derivatives and consist of linear chains of galactosyl residues, attached to the glucose moiety of sucrose via α-(1→6) glycosidic linkage [see review by Martinez-Villaluenga et al 2008]. The major RFO of lentil seeds include raffinose (trisaccharide), stachyose (tetrasaccharide) and verbascose (pentasaccharide). Large variations in the concentration of α-galactosides in lentil seeds ranging from 1.8 - 7.5% have been reported (Wang and Daun 2006; Martinez-Villaluenga et al 2008). Alpha-galactosidase enzyme is required to break α-(1→6) glycosidic linkages of α-galactosides to digest them. Humans and monogastric animals lack α-galactosidase enzymes and as a result RFO pass undigested through the stomach, and are fermented in the lower intestine by colonic microflora. This process of anaerobic fermentation produces carbon dioxide, methane, hydrogen and short chain fatty acids. Consumption of large quantities of RFO (RFO > 3 g^{-1}day) in food produce gases and may cause abdominal pain, diarrhea, discomfort and flatulence depending on individual sensitivity, thus restricting lentil consumption by humans (Cristofaro et al 1974; Price et al 1988; Tomomatsu 1994). High concentrations of RFO in food are therefore considered anti-nutritional and a reduction in RFO concentration and development of low RFO lentil cultivars is desired. However, non-digestible oligosaccharides including RFO, dietary fiber and resistant starch play an important role in promoting human health by acting as prebiotics which selectively stimulate growth of beneficial microorganisms such as *Bifidobacterium* and *Lactobacillu*s in the colon (Salminen et al 1998; Roberfroid 2002; Swennen et al 2006). Short chain fatty acids produced as a by product of the fermentation of the non-digestible oligosaccharides have been reported to play a role in the

prevention of colon cancer, reduction of inflammation and increases availability of minerals in the colon (Topping and Clifton 2001).

Lentil genotypes with increased protein, amylose and reduced RFO concentration will increase lentil quality and may increase lentil consumption by humans. The main objective of this study was to evaluate selected lentil genotypes for variation in seed composition and furthermore to determine association between major seed constituents.

3.3 Materials and Methods

3.3.1 Materials

Twenty-two lentil (*Lens culinaris* Medikus subsp. *culinaris*) genotypes including lentil genotypes, breeding lines and cultivars were grown in 2005 at the Preston Avenue plot area, University of Saskatchewan, Canada (Table 3.1). Each genotype was grown in four rows 4.6 m long and 0.3 m apart under rain-fed conditions with no fertilizer application. Mature lentil seeds were ground in a cyclone sample mill (Udy Corporation, Fort Collins, Colorado, USA) and the flour was used to determine total starch, protein and soluble carbohydrates concentration of lentil genotypes. Lentil seeds were manually decorticated and seed coats weighed.

3.3.2 Thousand-seed weight and seed coat colour

Thousand-seed weight was determined in grams (g) by counting 300 lentil seeds with two replications using an electronic seed counter (Seedburo Equipment Co., Chicago, IL, USA) and a balance. The seed coat colour of intact lentil seeds was recorded with Commission Internationale d'Eclairage (CIE) L*, a* and b* colour scale using a HunterLab miniScan XE plus colorimeter (Model 45/0-L, HAL, USA) having Xenon flash lamp as a light source. The colorimeter was calibrated with standard black and white calibration tiles provided with the instrument and colour was recorded in triplicate.

3.3.3 Total starch determination

Lentil total starch concentration was quantified for duplicate samples by an enzymatic assay method (McCleary et al 1994) using a commercial kit (Megazyme International Ireland Ltd, Wicklow Ireland) with two replications. Briefly, 100 mg lentil flour sample was suspended in 0.2 mL of 80% (v/v) ethanol and mixed vigorously using a vortex mixer. Thermostable α-amylase (3 mL, 300 U) in 3-(*N*-morpholino) propanesulfonic acid (MOPS, pH 7) buffer was

Table 3.1. Description of 22 lentil genotypes included in the study.

Genotype	Origin	Cotyledon Colour	Pedigree
CDC Robin	CDC, Canada	Red	CDC Matador//Eston/ESOR-3-6-1
CDC Redberry	CDC, Canada	Red	1049F3/819-5R
2670B	CDC, Canada	Red	1218D-18//TMP9586/TMP9586
ILL 1704	Ethiopia	Red	-
ILL 8006-BM4	Bangladesh	Red	
ILL 7537	Syria	Red	-
ILL 7502	Syria	Red	-
ILL 5684	Syria	Red	-
ILL 5588(Northfield)	Jordan	Red	-
PI 339283	Antalya, Turkey	Red	-
Eston	CDC, Canada	Yellow	PI 179307
Richlea	CDC, Canada	Yellow	Laird/PI 179310
Indianhead	CDC, Canada	Yellow	NEL 481
1294M-23	CDC, Canada	Yellow	804-3(Gorni Vadin/Araucana//578.28/Gorni Vadin)
1048-8R	CDC, Canada	Yellow	544-19/578-27
1156-2-17	CDC, Canada	Yellow	638-3// Lenea/639-38
964a-46	CDC, Canada	Yellow	638-2/89-12
PI 468901	Brazil	Yellow	-
PI 320937	Germany	Yellow	-
PI 345629	Ulyanovsk,USSR	Yellow	-
ILL 4605(Precoz)	Argentina	Yellow	-
ILL 6979	Unknown	Yellow	-

*CDC - Crop Development Center, University of Saskatchewan, Saskatoon, Canada

added to the sample and incubated at 100 °C for 8 min and mixed every 2 min to hydrolyse starch to dextrins. The sample was subsequently incubated with 4 mL of 20 mmol L^{-1} sodium acetate buffer (pH 4.5) and 0.1 mL of 20 U amyloglucosidase at 50 °C for 30 min to hydrolyse dextrins to glucose. The reaction mixture was diluted to 100 mL. To 1 mL aliquot, 3 mL of glucose determination reagent was added and incubated at 50 °C for 20 min. Total starch was calculated as free glucose by measuring the absorbance of quinoneimine dye at 510 nm (McCleary et al 1994). Commercial corn starch (Arancia Corn Products SA de CV, Mexico City, Mexico) provided with the Megazyme kit was used as a standard for starch concentration.

3.3.4 Starch extraction for amylose determination

Starch was isolated from lentil seeds in duplicate samples using the method described by Zhao and Sharp (1996) and Demeke et al (1997) with some modifications. Briefly, 100 mg of ground lentil flour was added to 1.5 mL of distilled water in a 2 mL microfuge tube and made into slurry by mixing thoroughly for 1 minute using a vortex. The slurry was centrifuged at 13 000 × *g* for 5 min and the supernatant was discarded. The starch pellet was dissolved in 200 µl water, layered on top of 1.5 ml 70% cesium chloride (CsCl) and centrifuged at 13 000 × *g* for 10 min and supernatant discarded to remove nonstarch impurities. The starch pellet was washed two times with 1 mL buffer (9.5 ml wash buffer and 500 µl β-mercaptoethanol), three times with 1 mL water and centrifuged at 13 000 × *g* for 5 min. The starch pellet was finally washed with 1 mL acetone, and centrifuged at 13 000 × *g* for 5 min. The supernatant was discarded and resulting dried starch was used for amylose concentration determination.

3.3.4.1 High performance liquid chromatography (HPLC) separation of amylose and amylopectin

The amylose and amylopectin concentration of lentil seeds was determined by high-performance size exclusion chromatography (HP-SEC) for duplicate samples (Demeke et al 1999). A 3 mg starch sample suspended in 3 mL of distilled water in a glass tube which was incubated at 130 °C for 30 min and mixed thoroughly every 10 min. The resulting gelatinised starch solution (1 mL) was debranched by transferring 55 µL of 1 M sodium acetate (pH 4) and 2.5 µl of isoamylase (200 U/mL) at 40 °C for 4 hr. The debranching reaction was stopped by placing tubes in boiling water for 20 min. The debranched starch solution was freeze-dried (Model 8 (75040), Labconco Corporation, Kansas City, USA). The freeze-dried starch sample

was dissolved in 200 µL dimethyl sulfoxide (DMSO, 99% v/v), centrifuged at 15 000 × *g* for 10 min and the supernatant was used for HPLC analysis. The HPLC system consisted of Waters autosampler and a Waters 410 differential refractometer (Waters Corporation, Milford, MA, USA) linked to a PLgel MiniMix column (4.6 mm i.d.) maintained at 90 °C (Polymer Laboratories, Inc., Amherst, MA, USA). DMSO (1M) was used as mobile phase at a flow rate of 0.2 mL min^{-1} with the sample run and delay time of 20 and 25 min, respectively. The data was collected and analysed using Empower software (Waters Corporation). The peak areas of long and short glucan chains were integrated and ratios calculated as amylose and amylopectin, respectively (Demeke et al 1999).

3.3.5 Determination of protein concentration

The protein concentration of lentil seed meal was determined by measuring the nitrogen concentration of lentil samples according to AACC Method 46-30 (AACC International 2000) in duplicate samples. Briefly, the sample (0.25 g) was pyrolized at 850 °C with pure oxygen and the products of combustion were passed through hot copper to remove oxygen and to convert NOx into N2, followed by lecosorb and anhydrone treatment for removal of carbon dioxide and water. The nitrogen in the sample was measured by a thermal conductivity cells (FP-528 Protein/Nitrogen Analyser, Leco Corporation, St Joseph, MI, USA). The crude protein concentration of each sample was calculated using the following formula:

Crude protein = N% × 6.25 (g)

3.3.6 Determination of RFO, sucrose and glucose concentration

The soluble carbohydrates of lentil seeds were determined using an enzyme based method (McCleary et al 2006) to hydrolyze sugars to glucose followed by colorimetric measurement of glucose using a raffinose/glucose assay kit (Catalogue no: K-RAFL, Megazyme International Ireland Ltd, Bray, Ireland). Soluble sugars such as RFO and sucrose were hydrolyzed with α-galactosidase (from *Aspergillus niger*) and invertase (from yeast) into D-glucose, D-galactose and D-fructose. D-glucose concentration was determined using glucose oxidase peroxidase reagent (GOPOD reagent). The concentration of raffinose, stachyose, verbascose and other higher homologues of the RFO in seed samples were measured as a group,

because α-galactosidase hydrolyzes all members of the RFO family. Since one mole of each of the RFO contains one mole of D-glucose, the concentrations are presented on a molar basis.

Lentil flour (0.5 ± 0.01g) was weighed into glass test tubes and 5 mL of 95% (v/v) ethanol was added to each tube. The lentil seed meal and ethanol mixture in a tube was incubated in a water bath for 5 min at 85 °C. After incubation, the contents of each tube were quantitatively transferred to individual 50 mL volumetric flasks and the volume was made up with sodium acetate buffer (50 mM, pH 4.5). The contents of the volumetric flasks were mixed thoroughly for 15 min at room temperature. After 15 min incubation 5 mL of the slurry was transferred to a glass tube, chloroform (2 mL) was added and the tube was centrifuged at 1000 × *g* for 10 min. Supernatant (0.2 mL) of each sample was added to 0.2 mL of sodium acetate buffer (50 mM, pH 4.5), 0.2 mL of invertase (8.3U/mL) and invertase plus α-galactosidase enzyme solution (invertase 8U/mL and α-galactosidase 40U/mL) in separate glass tubes. All solutions were incubated for 20 min at 50 °C. GOPOD reagent (3 mL) was added to all tubes including the reagent blank (0.4 mL sodium acetate buffer) and glucose standards (D-glucose 100 µg/0.1 mL) and all tubes were incubated for 20 min at 50 °C. The glucose concentration of the samples was determined by measuring the absorbance of quinoneimine dye at 510 nm using a spectrophotometer (DU 800, Beckman Coulter Inc). Glucose and sucrose concentration of the lentil samples are reported in g 100 g^{-1} lentil flour whereas RFO concentration is shown in mmoles 100 g^{-1}flour. The concentration of soluble sugars was calculated as follows:

Glucose = $\Delta A \times F \times 50$,

Sucrose = $(\Delta B - \Delta A) \times F \times 50$,

RFO = $(\Delta C - \Delta B) \times F \times 50$,

'F' is a factor to convert absorbance to µmoles of glucose and ΔA, ΔB and ΔC are the absorbance of sample plus sodium acetate buffer, sample plus invertase and sample plus invertase and α-galactocidase enzyme solution respectively.

3.3.7 Extraction of soluble carbohydrates for RFO profile analysis

Lentil seeds soluble carbohydrates were extracted with 80% (v/v) ethanol based on methods of Karoutis et al (1992), Frias et al (1994) and Sanchez –Meta et al (1998) with some modifications. Lentil flour (0.5 g) was intensively mixed with 10 ml of ethanol (80%, v/v) for 30 sec and incubated in a shaking water bath (Precision Scientific, Chicago, Illinois) for 45 min at

60 °C with vigorous mixing at 15 min intervals. The solution was then centrifuged at 12,100 *g* for 10 min and the supernatant was collected. The sample pellet was re-extracted with 10 mL of ethanol (80%, v/v) as previously described and the two supernatants were pooled. The extract (5 mL) was passed through a C18 (Solid Phase System Columns, Honeywell Burdick and Jackson™) cartridge using a vacuum manifold to remove proteins and lipids present in the extract. The final 2 mL extract was collected and an aliquot (1.6 mL) was freeze dried and used for composition analyses.

3.3.8 High performance liquid chromatography (HPLC) separation of RFO

Lentil seed RFO were separated by HPLC-SEC using a Sugar- Pak 1 column linked to a refractive index detector. Freeze dried samples were dissolved in 500 µL of nanopure filtered (0.2 µ) water and centrifuged at 15,700 *g* for 10 min. After centrifugation the samples were passed through nylon syringe filters (0.45 µm) (Phenomenex, Torrance, CA, USA) and injected using an autosampler into a Sugar- Pak 1 column (Ca^{2+} cation - exchange resin) maintained at 90 °C. Nano pure filtered water containing $CaNa_2$ EDTA (0.0001 M) was used as the mobile phase at a flow rate 0.5 mL min^{-1}. Sample run time and delay until the next injection was 20 min for each sample. RFO peaks in lentil samples were determined by comparing to retention times of pure raffinose and stachyose (Sigma) and verbascose (Fluka) standards. The peak area of verbascose, stachyose and raffinose in a sample was used to calculate individual RFO concentration in g 100 g^{-1}flour using standard curves (0.1 to 2.5 mg/ml concentration) of raffinose, stachyose and verbascose.

3.3.9 Statistical analyses

Analyses of variance (ANOVA) and correlation analysis were conducted using SAS version 9 (SAS Institute Inc, Cary, N.C). Duncan multiple range test (DMRT) was used to separate means and significance was accepted at $P \leq 0.05$.

3.4 Results and Discussion

3.4.1 Thousand-seed weight, seed coat colour of lentil genotypes

The analysis of variance showed significant ($P \leq 0.05$) variation in the seed weights of different lentil genotypes (Appendix 3.1). The thousand-seed weight of lentil genotypes ranged from 13.3 - 56.3 g 1000^{-1} seeds (Table 3.2). Genotype ILL 6976 and Indianhead, both with

Table 3.2. Thousand seed weight and seed coat colour of lentil genotypes.

Genotype	1000 seed weight (g)	L*	a*	b*
Red cotyledon genotypes				
CDC Robin	25.1^{m}	37.0^{ij}	7.9^{c}	10.6^{k}
CDC Redberry	33.3^{h}	39.3^{g}	7.7^{dce}	11.1^{j}
2670B	36.0^{f}	38.8^{g}	7.8^{dc}	16.2^{f}
ILL 1704	26.3^{l}	31.3^{l}	6.8^{hg}	7.8^{o}
ILL 8006-BM4	30.2^{k}	35.1^{k}	7.1f^{g}	8.9^{n}
ILL 7537	32.1^{i}	37.4^{ih}	7.3d^{fge}	9.5^{m}
ILL 7502	35.8^{f}	38.0^{h}	10.9^{a}	14.9^{g}
ILL 5684	50.5^{c}	42.2^{e}	8.8^{b}	14.4^{g}
ILL 5588(Northfield)	34.4^{g}	36.7^{j}	10.6^{a}	12.4^{i}
PI 339283	34.3^{g}	35.1^{k}	5.0^{j}	6.6^{p}
Yellow cotyledon genotypes				
Eston	25.5^{ml}	43.6^{c}	6.0^{i}	18.6^{d}
Richlea	45.5^{e}	44.9^{b}	6.4^{hi}	17.3^{e}
Indianhead	13.3^{o}	-	-	-
1294M-23	49.0^{d}	44.1^{f}	5.2^{j}	20.2^{b}
1048-8R	31.1^{j}	43.7^{c}	7.6^{dfce}	20.7^{a}
1156-2-17	35.8^{f}	45.0^{b}	7.7^{dce}	14.9^{g}
964a-46	52.9^{b}	42.4^{e}	8.5^{b}	19.1^{c}
PI 468901	25.7^{ml}	43.0^{d}	7.3^{dfge}	14.6^{g}
PI 320937	23.4^{n}	23.73^{m}	-0.3^{k}	-1.2^{q}
PI 345629	23.3^{n}	37.6^{h}	6.2^{i}	10.1^{l}
ILL 4605(Precoz)	53.4^{b}	46.9^{a}	7.1^{fg}	18.2^{d}
ILL 6979	56.3^{a}	41.0^{c}	7.1^{fg}	13.8^{h}
Range	13.3 - 56.3	23.7 - 46.9	-0.3 - 8.8	-1.2 - 20.7
Std Error	±0.3	±0.2	±0.2	±0.2

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$ based on DMRT.

yellow cotyledons, had significantly ($P \leq 0.05$) higher and lower thousand-seed weights of 56.3 g and 13.3 g, respectively. Among red cotyledon genotypes significantly ($P \leq 0.05$) higher and lower thousand-seed weights of 50.5 g and 25.1 g were recorded for genotype ILL 5684 and CDC Robin, respectively.Significant ($P \leq 0.05$) differences were also found in all colour parameters of seed coats for lentil genotypes. In the CIE system of colour representation the values L*, a*, and b* [(black (L* = 0) to white (L* = 100), Red (a* = +) to green (a*= -) and yellow (b* = +) to blue (b* = -) describe a uniform three-dimensional colour space**.** The L*, a*, and b* axis values for seed coats of different genotypes ranged from 23.7 - 46.9, -0.3 - 8.8 and -1.2 - 20.7, respectively, indicating differences in the darkness, redness and yellowness of the seed coats (Table 3.2). Based on L* axis values genotype ILL 4605 (Precoz) had the lightest whereas genotype PI320937 had the darkest seed coat colour. Similarly, a* values indicated that genotype ILL 7502 and ILL 5588 (Northfield) had no significant difference in the redness of seed coat colour, however, their seed coat colour was significantly ($P \leq 0.05$) different than other lentil genotypes. The negative a*, b* and low L* values of the genotype PI 320937 indicated a dark black blue seed coat colour for this lentil genotype. Similar variations in seed coat colour have been reported for other legumes including some lentil varieties (Xu et al 2007; Segev et al 2010). A major limitation of this method of colour determination, however, is its inability to differentiate between unspotted and spotted lentil seed coats.

3.4.2 Effect of decortication on starch concentration of lentil genotypes

Decortication of lentil seeds exhibited a significant ($P \leq 0.05$) effect on the starch concentration of lentil seeds (Table 3.3). The total starch concentration increased in the decorticated seeds and constituted 47.4 - 53.7 g 100 g^{-1} of seed dry matter compared to total starch concentration of 40.2 - 46.1 g 100 g^{-1} seed dry matter of lentil genotypes with seed coats (Table 3.4). This increase in starch concentration was expected because seed coats contain very little starch. A similar, increase in the starch concentration in decorticated lentils was reported for other lentil cultivars (Wang 2008; Wang et al 2009). The highest starch concentration in lentil seeds was recorded for Richlea. However, in Richlea decorticated seeds the starch concentration was not significantly different ($P \leq 0.05$) than the starch concentration of line 1048-8R. The increase in starch concentration in the decorticated seed samples corresponded to removed seed coat weight in some genotypes; however, in line 1048-8R, 1294M-23 and 964a-46, the increase

Table 3.3. Starch concentration of hulled and decorticated seeds and coat weights of lentil genotypes.

Genotypes	Starch (g 100 g^{-1}d.w.)			Seed coat (% seed wt.)
	Hulled seeds	Decorticated	Difference	
Red cotyledon lentils				
CDC Robin	42.7^{fde}	51.1^{ebdfc}	8.5	8.7
CDC Redberry	42.6^{fde}	52.0^{bdac}	9.4	8.3
2670B	43.1^{fbdec}	48.2^{ih}	5.1	7.5
ILL 1704	43.7^{bdec}	48.8^{ih}	5.1	8.8
ILL 8006-BM4	43.7^{bdec}	51.7^{ebdac}	8.0	8.1
ILL 7537	42.9^{fdec}	49.8^{egdfh}	7.0	8.1
ILL 7502	44.5^{bdac}	53.0^{ba}	8.5	7.7
ILL 5684	45.1^{ba}	52.5^{bac}	7.4	7.9
ILL 5588(Northfield)	45.0^{ba}	52.1^{bac}	7.1	9.1
PI 339283	43.3^{fbdec}	50.5^{egdfc}	7.3	7.1
Yellow cotyledon lentils				
Eston	41.5^{fg}	51.2^{ebdc}	9.7	9.9
Richlea	46.1^{a}	53.7^{a}	7.6	8.1
Indianhead	43.2^{fbdec}	51.1^{ebdfc}	7.9	8.7
1294M-23	45.1^{ba}	47.4^{i}	2.3	8.6
1048-8R	46.0a	49.7^{egfh}	3.7	8.3
1156-2-17	43.8^{bdec}	51.9^{ebdac}	8.1	7.7
964a-46	44.6^{bdac}	49.1^{gfih}	4.5	8.3
PI 468901	40.2^{g}	50.7^{egdfc}	10.5	9.9
PI 320937	43.2^{fbdec}	50.4^{egdfch}	7.2	8.0
PI 345629	41.8^{fge}	50.4^{egdfch}	8.6	6.5
ILL 4605(Precoz)	44.9^{bac}	51.7^{ebdac}	6.8	8.6
ILL 6979	44.7^{bdac}	52.4^{bac}	7.7	8.3
Range	40.2 - 46.1	47.4 - 53.7	2.3 - 9.7	6.5 - 9.9
Std Error	±0.61	±0.66		

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$ based on DMRT.

in starch concentration was not proportional to the weight of the removed seed coat. The seed coat weight of different genotypes accounted for 6.5 - 9.9% of the total seed weight (Table 3.3). Differences in seed coat weight of different lentil genotypes, however, were not significant ($P \leq 0.05$) (Appendix 3.1).

3.4.3 Total starch, amylose and protein concentration of lentil genotypes

The analyses of variance indicated significant differences ($P \leq 0.05$) in the total starch concentration of different lentil cultivars and lines (Appendix 3.1). The total starch concentration of lentil genotypes ranged from 39.4 - 45.3 g 100 g^{-1}flour dry weight (Table 5). These starch concentrations are in agreement with previously reported starch concentration for lentil cultivars (Vidal-Valverde and Frias 1992; Wang and Daun 2006; Wang et al 2009). The differences in total starch concentration were more pronounced in yellow cotyledon lentils as compared to red cotyledon lentils. Lentil genotype PI 468901 and Richlea, both with yellow cotyledons, had the lowest and highest starch concentration of 39.4 g and 45.3 g 100 g^{-1}, respectively. The starch concentration of Richlea, however, was not significantly different ($P \leq 0.05$) than the starch concentration of 1048-8R lentil genotype. Among red lentil genotypes, both CDC Redberry and CDC Robin had the lowest starch concentration of 41.8 g 100 g^{-1} whereas genotype ILL 5684 had the highest starch concentration of 44.2 g 100 g^{-1}on dry weight basis.

A significant variation ($P \leq 0.05$) was also observed in the amylose concentration of lentil genotypes (Appendix 3.1). The highest and lowest amylose concentration of 29.8 g and 34.0 g 100 g^{-1} was recorded for yellow cotyledon genotypes ILL 6979 and PI345629, respectively (Table 3.4). Among red cotyledon lentil genotypes, PI 339283 had the highest amylose concentration of 33.8 g 100 g^{-1} starch, whereas genotype ILL7502 had the lowest amylose concentration of 30.7 100 g^{-1} starch. This variation in lentil amylose concentration of 3.1 g and 3.7 g 100 g^{-1} starch for red and yellow cotyledon genotypes, respectively, though statistically significant is very limited in range. Amylose concentration of these lentil genotypes are similar to the reported lentil amylose concentration of 33.0 - 34.5 g, 30.1 - 34.4 g and 30.6 - 33.9 g 100 g^{-1}starch reported by Hoover and Manuel (1995), Yoshimi and Toshiko (2006) and Kaur et al (2010), respectively.

Protein concentration of lentil genotypes also varied significantly ($P \leq 0.05$) and ranged from 23.8 - 29.3 g 100 g^{-1}flour (Appendix 3.1 and Table 3.4). The highest protein concentration

Table 3.4. Total starch, amylose and protein concentrations of selected lentil genotypes.

Genotype	Starch (g 100 g^{-1}d.w.)	Protein (g 100 g^{-1}d.w.)	Amylose (g 100 g^{-1}starch)
	Red cotyledon genotypes		
CDC Robin	41.8^{edc}	27.1^{e}	33.1^{bac}
CDC Redberry	41.8^{edc}	25.5^{l}	33.7^{a}
2670B	42.3^{bedc}	26.6^{fgh}	32.8^{bdc}
ILL 1704	42.8^{bdc}	29.3^{a}	33.1^{ba}
ILL 8006-BM4	42.8^{bde}	26.1ji	33.5^{ba}
ILL 7537	42.0^{edc}	27.6^{d}	33.5^{ba}
ILL 7502	43.6^{bac}	26.8^{f}	30.7^{h}
ILL 5684	44.2^{ba}	26.8^{f}	30.9^{hg}
ILL 5588(Northfield)	44.1^{ba}	25.8^{lk}	31.2^{hgf}
PI 339283	42.4^{bedc}	26.8^{fe}	33.8^{a}
	Yellow cotyledon genotypes		
Eston	40.7^{fe}	26.7^{fg}	31.8^{egf}
Richlea	45.3^{a}	23.8^{n}	31.9^{ef}
Indian Head	42.3^{bedc}	25.0^{m}	33.5^{ba}
1294M-23	44.2^{ba}	26.5^{gh}	31.7^{egf}
1048-8R	45.1^{a}	23.8^{n}	32.2^{edc}
1156-2-17	43.0^{bdc}	25.9^{jk}	31.2^{ef}
964a-46	43.7^{bac}	25.0^{m}	31.4^{ehgf}
PI 468901	39.4^{f}	28.0^{c}	32.0^{edf}
PI 320937	42.4^{bedc}	28.4^{b}	31.4^{ehgf}
PI 345629	41.0^{fed}	28.2^{bc}	34.0^{a}
ILL 4605(Precoz)	44.1^{ba}	25.8^{lk}	33.1^{ba}
ILL 6979	43.8^{bac}	26.3^{ih}	29.8^{i}
Range	39.4 - 45.3	23.8 - 29.3	29.8 - 34.0
Std Error	±0.61	±0.09	±0.28

Means followed by the same letter within a column are not significantly different at $P \leq 0.05$ based on DMRT.

of 29.3 g 100 g^{-1}flour was recorded for genotype ILL 1704 (red cotyledon) followed by 28.4 g 100 g^{-1}flour protein concentration for genotype PI 320937 (yellow cotyledon). The protein concentration of genotype PI 320937, however, was not significantly different than genotype PI 345629. A significantly lower ($P \leq 0.05$) protein concentration of 23.8 g 100 g^{-1}flour was recorded for both Richlea and genotype 1048-8R. The mean protein concentration of red 100 g^{-1} cotyledon lentil genotypes of 26.8 g was slightly higher than yellow cotyledon lentils mean protein concentration of 26.1 g 100 g^{-1}flour. Variation in protein concentration of 4.6 g 100 g^{-1}flour among yellow cotyledon genotypes was relatively large compared to variation of 3.8 g 100 g^{-1}flour protein concentration observed in red cotyledon genotypes. The values of protein concentration of lentil genotypes obtained in this study are in range of protein concentration of 21.4 - 30.2 g 100 g^{-1}flour reported for several lentil varieties (Wang and Daun 2006; Wang et al 2003).

Trait correlation analysis indicated a significant ($P \leq 0.05$) positive association ($r = 0.6$) between thousand-seed weight and seed starch concentration of lentil genotypes (Table 3.5). A significant ($P \leq 0.05$) negative correlation was also found between thousand seed weight and amylose ($r = -0.5$) as well as thousand seed weight and protein concentration ($r = -0.3$) of lentil genotypes. Though, large variation existed for thousand seed weight with in yellow and red cotyledon lentil genotypes, higher thousand seed weights were observed for several yellow cotyledon lentil genotypes. Genotype ILL 5684 (red cotyledon) and CDC Indianhead (yellow cotyledon) with higher and lower thousand seed weights of 50.5 g and 13.3 g, respectively, were rather exceptions for their respective groups. Correlation analysis also indicated a significant ($P \leq 0.05$) negative association between starch and protein ($r = -0.5$) and starch and amylose concentration ($r = -0.3$). No significant correlation existed between amylose and protein concentrations in lentil genotypes (Table 3.5).

Although no striking variations in the total starch, amylose and protein concentration were observed in lentil genotypes studied here, genotype PI 345629, a yellow cotyledon genotype originating from Ulyanovsk, Russia, was found to be an interesting genotype with respect to its low total starch, high amylose and moderately high protein concentration. Though, the selection and development of a cultivar depends on its end use, this genotype may be helpful in development of lentil cultivar for consumers with preference for low bioavailable and low glycemic index starches.

Table 3.5. Correlation coefficients among different traits in seeds of lentil genotypes.

	Seed wt.	Seed coat wt.	Starch	Amylose	Protein	RFO	Sucrose	Glucose
Seed wt.	1	-0.1^{ns}	0.6^{**}	-0.5^{**}	-0.3^{*}	0.4^{*}	0.3^{*}	0.1^{ns}
Seed coat wt.		1	0.2^{ns}	-0.1^{ns}	-0.3^{ns}	0.1^{ns}	0.2^{ns}	-0.1^{ns}
Starch			1	-0.3^{*}	-0.5^{**}	-0.1^{ns}	0.1^{ns}	-0.2^{ns}
Amylose				1	0.1^{ns}	-0.3^{*}	-0.1^{ns}	-0.2^{ns}
Protein					1	0.1^{ns}	-0.1^{ns}	-0.3^{ns}

**, * and ns= significant at $P \leq 0.01$, $P \leq 0.05$ and not significant, respectively.

3.4.4 Soluble carbohydrates concentration and composition of lentil genotypes

Variation in the total RFO and sucrose concentration in the seeds of different lentil genotypes was significant ($P \leq 0.05$) and ranged from 4.6 - 6.6 mmoles and 0.7 - 2.4 g 100 g^{-1}flour, respectively (Table 3.6). Differences in glucose concentration, however, were not significant ($P \leq 0.05$) and ranged from 0.04 - 0.08 g 100 g^{-1}flour. These sugar concentration agree closely with reported sucrose and glucose concentration range of 1.1 - 3.0 and 0.01 - 0.30 g 100 g^{-1} dry matter (Frias et al 1995, 1996; Wang and Daun 2006) and total RFO concentration of 4.5 - 5.5 mmoles 100 g^{-1}flour for several lentil cultivars (Tahir et al 2011a). Lentil genotypes, PI 468901 and Indianhead, both with yellow cotyledon, had the highest and the lowest total RFO concentration, respectively. Genotype PI 468901 total RFO concentration of 6.6 mmoles 100 g^{-1} flour, however, was not significantly different than the red cotyledon lentil genotype ILL 8006-BM4 total RFO concentration of 6.5 mmoles 100 g^{-1}flour. Among red cotyledon genotypes the RFO concentration ranged from 5.2 - 6.4 mmoles 100 g^{-1}flour. The variation in total RFO concentration in different lentil genotypes though statistically significant ($P \leq 0.05$) was limited (Table 3.6). This narrow range of variation may be due to the fact that all these genotypes belong to *Lens culinaris ssp. culinaris* and may have a common genetic background. It may also be the case that limited natural genetic variation exists for RFO concentration in lentils. Analysis of wild species and subspecies of lentil may, therefore, give a better insight on natural genetic variation for RFO concentration in lentils.

The sucrose concentration of 2.4 g 100 g^{-1}flour of lentil genotype ILL 7537 was significantly ($P \leq 0.05$) higher than all lentil genotypes followed by breeding line 1048-8R sucrose concentration of 2.2 g 100 g^{-1}flour. The lowest ($P \leq 0.05$) sucrose concentration of 0.7 g 100 g^{-1}flour was recorded for lentil cultivar Indianhead. Correlation analysis showed a significant ($P \leq 0.05$) inverse association ($r = -0.3$) between RFO and amylose concentration. A non significant ($P \leq 0.05$) correlation ($r = -0.1$) was also found between total RFO and starch concentration in seeds of different genotypes. Similarly, relationship between RFO and protein concentration ($r = 0.1$) was non-significant ($P \leq 0.05$) (Table 3.5).

Composition analysis of RFO showed significant variation ($P \leq 0.05$) in the individual RFO profiles of different lentil genotypes. Stachyose was the most abundant RFO in lentil flour,

followed by raffinose and verbascose. The stachyose concentration of lentil genotypes ranged from 1.7 - 2.9 g 100 g^{-1}flour (Table 3.6). This range of stachyose is in close agreement with reported range of stachyose concentration of 1.1 - 3.7 g 100 g^{-1}dry matter for lentil genotypes (Frias et al 1994; Frias et al 1996). Both genotype 1294M-23 and Eston had the highest stachyose concentration of 2.9 g 100 g^{-1}flour, followed by line ILL 6979 with stachyose concentration of 2.7 g 100 g^{-1}flour. Line 1156-2-17 stachyose concentration of 1.7 g 100 g^{-1}flour was significantly ($P \leq 0.05$) lower than all lentil genotypes. The raffinose concentration of lentil genotypes ranged from 1.6 - 2.4 g 100 g^{-1}flour. The highest raffinose concentration was observed in line 1048-8R whereas the lowest raffinose concentration was recorded for lentil line 1156-2-17. Similarly, variation in verbascose concentration ranged from 1.2 - 1.9 g 100 g^{-1} flour. Line 964a-46 concentration of verbascose of 1.9 g 100 g^{-1}flour was significantly higher than all other lentil genotypes. Indianhead, CDC Redberry, ILL 5684 and 1156-2-17 all shared the lowest verbascose concentration of 1.2 g 100 g^{-1}flour. Variation in the individual RFO concentration of these lentil genotypes though statistically significant was limited in range and in close agreement with reported raffinose and verbascose concentration for lentil cultivars (Vidal-Valverde et al 1993a, 1993b; Tahir et al 2011a). The total oligosaccharide concentration was 4.5 - 6.7 g 100 g^{-1}flour in different lentil genotypes (Table 3.6) is in close agreement with reported values of 5.4 - 5.9 g 100 g^{-1}flour for several lentil varieties (Tahir et al 2011a). Lentil breeding line 1156-2-17 originating from Canada had the lowest collective raffinose, stachyose and verbascose concentration of 4.5 g 100 g^{-1}flour followed by ILL 5684, CDC Redberry and Indianhead with concentration of 4.9, 5.1 and 5.3 g 100 g^{-1}flour, respectively. It may be mentioned that for lentil genotypes 1156-2-17, CDC Redberry and Indianhead, lower total RFO concentration were obtained using the enzyme based colorimetric method. These genotypes may therefore be helpful in the study and development of low RFO lentil cultivars.

Table 3.6. Soluble carbohydrate concentration of selected lentil genotypes.

Genotypes	RFO	Sucrose	Glucose	Raffinose	Stachyose	Verbascose	Total
	(mmoles 100 g^{-1}flour)			(g 100 g^{-1}flour)			
			Red cotyledon lentils				
CDC Robin	6.1^{ebdac}	1.4^{ji}	0.04^{dc}	2.2^{bac}	2.4^{ebdac}	1.4^{cbd}	6.0
CDC Redberry	5.6^{ehdgf}	1.9^{dc}	0.05^{bdc}	2.0^{ebdac}	1.9^{ed}	1.2^{cd}	5.1
2670B	5.5^{hgf}	2.0^{dc}	0.08^{a}	2.0^{bdac}	2.6^{bdac}	1.4^{cbd}	6.0
ILL 1704	5.3^{hg}	1.7^{fe}	0.04^{dc}	2.3^{ba}	2.7^{bac}	1.5^{cbd}	6.5
ILL 8006-BM4	6.5^{a}	1.3^{j}	0.04^{dc}	2.2^{bdac}	2.5^{bdac}	1.5^{cbd}	6.2
ILL 7537	5.2^{h}	2.4^{a}	0.04^{d}	1.9^{ebdc}	2.5^{bdac}	1.4^{cbd}	5.8
ILL 7502	6.1^{ebdac}	1.7^{f}	0.05^{bdac}	1.9^{ebdc}	2.5^{bdac}	1.4^{cbd}	5.8
ILL 5684	5.9^{ebdgcf}	2.0^{c}	0.05^{bdac}	1.8^{ed}	1.9^{ed}	1.2^{cd}	4.9
ILL 5588(Northfield)	5.9^{ebdcf}	1.5^{hgi}	0.04^{dc}	2.0^{bdac}	2.7^{bac}	1.3^{cbd}	6.0
PI 339283	6.0^{ebdacf}	1.5^{hg}	0.05^{bdac}	2.1^{bdac}	2.4^{bdac}	1.5^{b}	6.0
			Yellow cotyledon lentils				
Eston	6.4^{bac}	2.0^{dc}	0.08^{ba}	2.2^{ba}	2.9^{a}	1.5^{cbd}	6.6
Richlea	5.8^{ehdgcf}	1.5^{hgi}	0.05^{bdac}	2.1^{bdac}	2.3^{ebdac}	1.4^{cbd}	5.8
Indianhead	4.6^{i}	0.7^{l}	0.06^{bdac}	2.1^{bdac}	2.0^{edc}	1.2^{cd}	5.3
1294M-23	6.3^{bac}	1.9^{de}	0.06^{bdac}	2.3^{ba}	2.9^{a}	1.5^{cbd}	6.7
1048-8R	5.6^{ehdgf}	2.2^{b}	0.06^{bdac}	2.4^{a}	2.8^{ba}	1.5^{cbd}	6.6
1156-2-17	5.5^{ehgf}	1.7^{fe}	0.07^{bac}	1.6^{e}	1.7^{e}	1.2^{cd}	4.5
964a-46	6.5^{ba}	1.6^{fg}	0.07^{bac}	2.1^{edc}	2.5^{ebda}	1.9^{a}	6.5

Genotypes	RFO	Sucrose	Glucose	Raffinose	Stachyose	Verbascose	Total
	(mmoles 100 g^{-1}flour)			(g 100 g^{-1}flour)			
PI 468901	6.6^{a}	1.5^{hgi}	0.07^{bdac}	2.2^{bdac}	2.4^{bdac}	1.6^{b}	6.2
PI 320937	5.9^{ebdcf}	1.0^{k}	0.04^{d}	2.2^{bdac}	2.2^{edc}	1.3^{cd}	5.7
PI 345629	5.8^{ehdgcf}	1.3^{j}	0.06^{bdac}	2.2^{bcac}	2.4^{ebdac}	1.4^{cbd}	6.0
ILL 4605(Precoz)	$6.0e^{bdacf}$	1.6^{fg}	0.05^{bdac}	1.9^{ebdc}	2.5^{bdac}	1.4^{cbd}	5.8
ILL 6979	6.2^{bdac}	1.4^{hji}	0.07^{bac}	2.1^{bdac}	2.7^{ba}	1.5^{cbd}	6.3
Range	4.6 - 6.6	0.7 - 2.4	0.04 - 0.08	1.6 - 2.4	1.7 - 2.9	1.2 - 1.9	4.5 - 6.7
Std Error	±0.18	±0.05	±0.01	0.1 - 0.2	0.2 - 0.4	0.1	

Means followed by the same letter within a column are not significantly different at $P < 0.05$ based on DMRT.

3.4.5 Summary and Conclusions

The seed constituents of different lentil genotypes showed significant differences in total starch, amylose, protein, total RFO, sucrose, glucose, raffinose, stachyose and verbascose concentration. Thousand seed weight and seed coat colour of different genotypes also varied significantly. Decortication increased starch concentration significantly; however, variation in seed coat weight of lentil genotypes was not significant. A significant negative correlation was found between starch and protein concentration of lentil seeds. Similarly, a significant inverse relationship was observed between thousand seed weight and protein concentration. The seed coat colour and the cotyledon colour had no association with starch and protein concentration of lentil seeds. Thousand seed weight of lentil genotypes was significantly positively correlated with the following traits: seed starch, total RFO and sucrose concentration. Association between RFO and starch concentration was not significant. Information obtained from this study may be helpful in understanding the relationship among different quality parameters and in development and selection of new lentil cultivars with increased starch, amylose, protein and reduced RFO concentration.

4 INFLUENCE OF ENVIRONMENT ON SEED SOLUBLE CARBOHYDRATES IN LENTIL CULTIVARS

4.1 Abstract

High concentration of raffinose family oligosaccharides (RFO) in lentils cause stomach discomfort and reduce its quality for human consumption. To develop strategies for lentil quality improvement we investigated variability, heritability and effects of environmental conditions on the concentration and composition of soluble carbohydrates in lentil seeds. Analyses of variance showed that cultivar, environment and their interaction had significant effects on the sugar concentrations in lentil seed. Glucose and sucrose concentrations of lentil cultivars ranged from 0.02 - 0.06 g and 1.22 - 1.67 g 100 g^{-1}flour, respectively. Total RFO concentration of lentil cultivars ranged from 4.5 - 5.5 mmoles 100 g^{-1} flour. In all lentil cultivars, RFO concentration was positively correlated with glucose and sucrose concentrations. Analysis of RFO profiles by high performance size exclusion chromatography (HP-SEC) showed that stachyose was the major RFO in all lentil cultivars followed by raffinose and verbascose, respectively. The broad sense heritability of sucrose and RFO estimated from the analyses of variance components was 0.89 and 0.85, respectively. Lentil seed RFO concentration is a highly heritable trait, thus making it amenable to genetic improvement to meet the consumer demands.

4.2 Introduction

Lentil (*Lens culinaris* Medikus subsp. culinaris) (2n = 2x = 14) is an important and inexpensive source of carbohydrate and protein for the human diet. High quantities of raffinose family oligosaccharides (RFO) in lentils cause stomach discomfort and thus reduce its human consumption. Lentil soluble carbohydrates constitute about one-tenth of the mature seed dry weight (Frias et al 1996), with sucrose and RFO as its major components (Vose et al 1976). RFO are sucrose-based carbohydrates consisting of linear chains of galactose residues attached to the glucose moiety of sucrose with α-(1→6) glycosidic linkages (Avigad and Dey 1997). Raffinose, stachyose and verbascose are the major RFO components with other higher homologues also found in some plant species (Dey 1985). These oligosaccharides have been associated with plant

responses to abiotic stresses and accumulate during cold acclimation (Gilmour et al 2000) or during seed desiccation (Obendorf 1997). Recently, Nishizawa et al (2008a) reported that raffinose and galactinol scavenge hydroxyl radicals, thus protecting plants from oxidative damages caused by environmental stresses. During seed germination, α-galactosidase hydrolyses RFO (Dey 1985) to provide energy and substrates for growth of the developing seedling (Peterbauer and Richter 2001). Blöchl et al (2007) showed that the inhibition of RFO breakdown during germination reduced this process in pea seeds, confirming the role of RFO as an energy source during seed germination.

Environmental factors affect the composition and quantity of soluble carbohydrates in plants, as they do with other seeds storage components. A three-fold increase in stachyose concentration was observed in buckwheat seed embryos grown at low temperatures (18 °C) compared to embryos grown at higher temperatures (25 °C) (Horbowicz et al 1998). Similarly, sucrose and RFO, particularly raffinose concentration, increased in *Xerophyta viscose* (family Vellociaceae) leaves under water deficit conditions (Peters et al 2007).

In pulses, RFO concentration range from 2 to 10 g 100 g^{-1} of dry matter (Guillon and Champ 2002). In several cultivars of lentils large differences in the α-galactosides concentration ranging from 1.8 to 7.5% of seed dry weight have been reported (Martinez-Villaluenga et al 2008). These soluble carbohydrates are considered anti-nutritional because humans and mono-gastric animals lack α-galactosidase enzymes and thus cannot digest RFO. The RFO and α-galactoside fermentation in the colon by anaerobic bacteria produces carbon dioxide, hydrogen and methane gases along with short chain fatty acids (Price et al 1988). These gases produce flatus, and result in diarrhea, abdominal pain and discomfort (Fleming 1981). The undesirable effects of RFO limit lentil consumption by humans. Therefore, reducing the concentration of these soluble carbohydrates in lentil is highly desirable to promote human lentil consumption. Several methods such as soaking (Ogun et al 1989), addition of enzyme (Price et al 1988), germination (Vidal-Valverde et al 2002) and partial fermentation of pulses to reduce RFO concentration are time consuming and result in reduction of carbohydrates and energy.

Developing lentil cultivars with reduced RFO concentration will decrease the anti-nutritional properties, improve lentil quality as food, and promote human lentil consumption. As a first step to develop lentils with reduced RFO concentration, we examined the genetic

variability, heritability and effect of environment on the concentration and composition of lentil seed soluble carbohydrates.

4.3 Material and Methods

4.3.1 Materials

Eleven lentil (*Lens culinaris* Medikus ssp. *culinaris*) cultivars (Table 4.1) were grown in a randomized complete block design with three replications in two environments in 2004 and eight environments in 2005 in Saskatchewan, Canada (Table 4.2). Each cultivar was grown in four rows 4.6 m long and 0.3 m apart under rain-fed conditions with no fertilizer application. The pre-emergence herbicide imazethapyr was used (Pursuit, BASF Canada Inc.) to control weeds. The harvested lentil seed was cleaned and ground with a cyclone sample mill to produce a flour (Udy Corporation, Fort Collins, Colorado, USA). The flour was used for determining total soluble carbohydrate concentration and for RFO profile analyses.

4.3.2 Determination of glucose, sucrose and RFO concentrations

The soluble carbohydrate concentration of lentil seed meals were determined using an enzyme based method (McCleary et al 2006) to hydrolyze sucrose and α-galactosides to glucose followed by colorimetric measurement of glucose using a raffinose/glucose assay kit (Catalogue no: K-RAFL, Megazyme International Ireland Ltd, Bray, Ireland).

4.3.3 Extraction of soluble carbohydrates and RFO composition analysis

Lentil seeds soluble carbohydrates were extracted in duplicate samples with 80% (v/v) ethanol and separated by high performance size exclusion chromatography (HPLC-SEC) using a Sugar- Pak 1 column linked to a refractive index detector as reported.

4.3.4 Statistical analyses

Analyses of variance (ANOVA) and correlation analyses were conducted using SAS version 9 (SAS Institute Inc, Cary, N.C). Levene's test (Levene 1960) for equality of variances demonstrated the homogeneity of variances ($P > 0.5$) and normality of the data. Duncan multiple range tests were used to separate means and significance was accepted at $P \leq 0.05$. Broad sense heritability of soluble carbohydrates in lentil cultivars was estimated from the components of analyses of variance (Singh et al 1993) as follows:

$h^2 = \sigma^2_G / \{\sigma^2_G + \sigma^2_{GE} /l + \sigma^2_e/rl\}$, where σ^2_G and σ^2_{GE} represent cultivars and cultivars-by-environmental variances, while r and l represent replications and locations respectively.

4.4 Results and Discussion

4.4.1 Effect of environment on lentil seed glucose and sucrose concentration

The glucose concentration of lentil cultivars ranged from 0.02 - 0.06 g 100 g^{-1} flour. The mean glucose concentration for all selected cultivars was 0.03 g 100 g^{-1}flour (Table 4.1). Sucrose concentration of lentil cultivars ranged from 1.2 - 1.7 g 100 g^{-1} flour with a mean concentration of 1.4 g 100 g^{-1} flour for all cultivars. The glucose and sucrose concentrations observed in this study concur with previously reported lentil seed concentrations of glucose (Bhatty and Christison 1984) and sucrose (Vidal-Valverde and Frias 1992; Vidal-Valverde et al 1993a).

The analysis of variance (Appendix 4.1) showed strong and significant ($P \leq 0.0001$) cultivar and environmental effects on glucose, sucrose and total RFO concentrations in lentil seeds. The effects of the interaction between cultivar and environmental conditions on soluble carbohydrates were also significant. CDC Vantage had significantly higher ($P \leq 0.05$) mean glucose concentration of 0.06 g 100 g^{-1}flour among all cultivars. CDC Rosetown had the lowest glucose concentration (0.02 g 100 g^{-1}flour) among all cultivars (Table 4.1). CDC Rosetown glucose concentration, however, was not significantly different ($P \leq 0.05$) from CDC Rouleau and CDC Robin glucose concentration. The glucose concentrations of lentil cultivars varied significantly over different environments. The mean glucose concentration of the lentil cultivars grown at Goodale 2004 (0.07 g 100 g^{-1}flour) was significantly ($P \leq 0.05$) higher than all other environments (Appendix 4.2). The lowest mean glucose concentrations were observed in lentil cultivars grown at Davidson 2004 (0.01 g 100 g^{-1}flour). However, the glucose concentrations of lentil cultivars at this environment were not significantly different than glucose concentrations at Scott 2005 and Kyle 2005.

The sucrose concentration of lentil seeds also varied with cultivar. On average the green cotyledon lentil CDC Sovereign had the highest and the red cotyledon lentil CDC Robin had the lowest sucrose concentration of 1.7 g 100 g^{-1} and 1.2 g 100 g^{-1}flour, respectively (Table 4.1). Duncan's multiple range tests showed that lentil cultivars varied significantly in sucrose concentration and fell into three groups. CDC Sovereign was the sole member of the high

Table 4.1. Seed soluble carbohydrates (100 g^{-1} flour) of lentil cultivars.

Cultivars	Cotyledon Colour	Glucose (g)	Sucrose (g)	Total RFO (mmoles)
CDC Glamis	Green	0.03	1.2	5.2
CDC Sovereign	Green	0.03	1.7	5.5
CDC Richlea	Green	0.04	1.4	5.1
CDC Vantage	Green	0.06	1.3	5.4
CDC Meteor	Green	0.04	1.3	5.2
CDC Milestone	Green	0.03	1.5	4.9
CDC Viceroy	Green	0.03	1.4	4.8
CDC Robin	Red	0.02	1.2	4.8
CDC Redberry	Red	0.03	1.6	4.5
CDC Rouleau	Red	0.02	1.6	5.3
CDC Rosetown	Red	0.02	1.3	4.6
Mean		0.03	1.4	5.0
Range		0.02 - 0.06	1.2 - 1.7	4.5 - 5.5
SE of Means		0.01	0.02	(±0.1)

sucrose group (1.7 g 100 g^{-1}flour), whereas five cultivars i.e. CDC Robin, CDC Meteor, CDC Glamis, CDC Vantage, and CDC Rosetown constituted the low sucrose (1.2 -1.3 g 100 g^{-1}flour) concentration cultivars group. The other five cultivars had medium sucrose concentrations (1.4 - 1.6 g 100 g^{-1}flour). Differences in sucrose concentration of lentil cultivars grown in different environments were also significant ($P \leq 0.05$). On average sucrose concentrations of lentil cultivars at Rosthern 2005 and Swift Current 2005 were significantly ($P \leq 0.05$) higher and lower, respectively, compared to the other environments (Appendix 4.2).

4.4.2 Lentil seed total RFO concentration

The total RFO concentration of lentil cultivars ranged from 4.5 - 5.5 mmoles 100 g^{-1} with a mean concentration of 5.0 mmoles 100 g^{-1}flour (Table 4.1). Comparison of the total RFO concentrations of lentil cultivars obtained in this study using the α-galactosidase mediated hydrolysis method (McCleary et al 2006) with lentil RFO concentrations reported in literature in grams is difficult because the α-galactosidase mediated hydrolysis method measures total RFO as a group instead of quantifying RFO concentrations individually in grams. The α-galactosidase mediated hydrolysis does not differentiate among individual RFO members and hence the total RFO concentration is representative of all oligosaccharides hydrolyzed by the enzyme. HPLC based methods for RFO determination requires considerable sample preparation for removal of lipids. Overlapping of elution peaks and different detector responses may also make quantification difficult (McCleary et al 2006). Although the use of a specific method depends on the objective of the study, the colorimetric method is less time consuming, quantitative and can be used for analyzing a large numbers of samples.

The variation in RFO concentration of the selected lentil cultivars though statistically significant ($P \leq 0.05$), were not large (Table 4.1). This may be due to the fact that these cultivars share a common genetic background. Duncan's multiple range tests, however, showed two distinct groups of lentil cultivars with contrasting RFO concentrations. The first group comprised of high RFO and included two sub groups. The first sub group (5.3 - 5.5 mmoles 100 g^{-1} flour) is comprised of CDC Sovereign, CDC Vantage and CDC Rouleau, whereas the second sub group (5.1 - 5.2 mmoles 100 g^{-1} flour) consisted of CDC Glamis, CDC Meteor and CDC Richlea. CDC Sovereign had a significantly higher ($P \leq 0.05$) RFO concentration than other cultivars in this group. The second group consisted of cultivars with the lowest mean RFO

Table 4.2. Precipitation (mm) at different environments during lentil growing season.

Environments	Soil Zone	May	June	July	Aug	Sep	Total
Kyle 2005	Brown	57.4	165.0	21.3	30.6	117.9	382.2
Rouleau 2005	Dark Brown	52.5	92.5	34.5	39.5	17.5	236.5
Rosthern 2005	Dark Brown	30.5	110.0	55.0	60.5	78.0	334.0
Swift Current 2005	Brown	29.0	96.5	27.0	49.0	50.0	251.5
Scott 2005	Dark Brown	50.7	88.2	60.4	71.5	93.5	364.3
Davidson 2005	Dark Brown	73.0	157.2	43.8	40.2	79.7	393.7
Davidson 2004	Dark Brown	20.0	58.0	57.0	119.4	22.5	276.9
Goodale 2004	Dark Brown	31.0	78.0	72.5	77.0	21.5	280.0
Goodale 2005	Dark Brown	30.5	110.0	55.0	60.5	78.0	334.0
SPG 2005	Dark Brown	30.5	110.0	55.0	60.5	78.0	334.0

Source (http://www.theweathernetwork.com/)

(4.5 - 4.9 mmoles 100 g^{-1}) and included CDC Redberry, CDC Rosetown, CDC Viceroy, CDC Robin and CDC Milestone. In this group of cultivars CDC Redberry had the lowest RFO concentration of all cultivars followed by CDC Rosetown, although the two were not significantly different.

4.4.3 RFO profile analysis of lentil seeds

HPLC-SEC analysis indicated a significant cultivar, environmental and cultivar × environmental interaction effects on RFO profiles in lentil seeds (Appendix 4.2; Fig. 4.1). Stachyose was the major RFO in all lentil cultivars followed by raffinose and verbascose (Fig. 2). Individual RFO profiles of lentil cultivars remained the same in all environments (Fig. 4.3).

The stachyose, raffinose and verbascose concentration of lentil cultivars ranged from 2.20 - 2.55, 1.95 - 2.22 and 1.15 - 1.33 g 100 g^{-1}flour, respectively (Fig. 4.2). These individual RFO concentrations of lentil cultivars are in close agreement with stachyose, raffinose and verbascose concentrations of 1.1 - 3.1, 0.16 - 1.49 and non detectable - 1.35 g 100 g^{-1} dry matter, respectively, reported for lentil seeds in the literature (Frias et al 1994, 1995, 1996; Vidal-Valverde et al 1993a, 1993b; Reddy et al 1984). Among cultivars, CDC Rouleau had a significantly higher ($P \leq 0.05$) stachyose concentration and significantly lower ($P \leq 0.05$) verbascose concentration of 2.55 and 1.17 g 100 g^{-1}flour, respectively, than all other cultivars. CDC Rouleau's raffinose concentration of 2.22 g 100 g^{-1}flour was also higher than all other cultivars. Across different environments the stachyose, raffinose and verbascose concentration ranged from 1.82 - 2.72, 1.70 - 2.44 and 1.16 - 1.40 g 100 g^{-1}flour, respectively (Fig. 4.3).

4.4.4 Environment influences RFO concentration in lentil genotypes

The differences in the RFO concentrations among cultivars may be due to the difference in RFO biosynthetic enzymes, specifically their affinity for substrates. A comparative study of the enzymes of the RFO biosynthetic pathway in lentil genotypes with varying RFO concentrations has not been reported to date. The total RFO concentration of lentil cultivars varied significantly over different environments (Appendix 4.1; Table 4.3). Based on Duncan's multiple range tests, environments can be divided into two different groups. The first group is composed of environments where high RFO concentrations were produced and consists of Goodale 2004 and 2005, Rouleau 2005, Swift Current 2005, Scott 2005, SPG 2005 and Rosthern

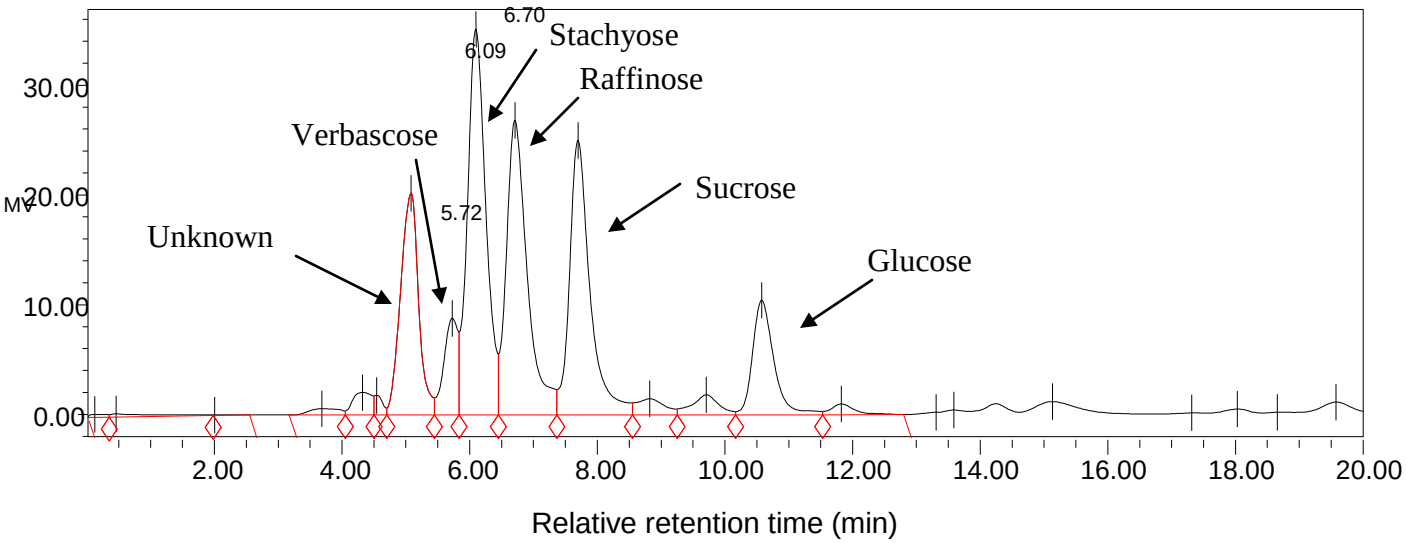

Figure 4.1. Representative HP-SEC analyses profile of lentil seed soluble carbohydrates.

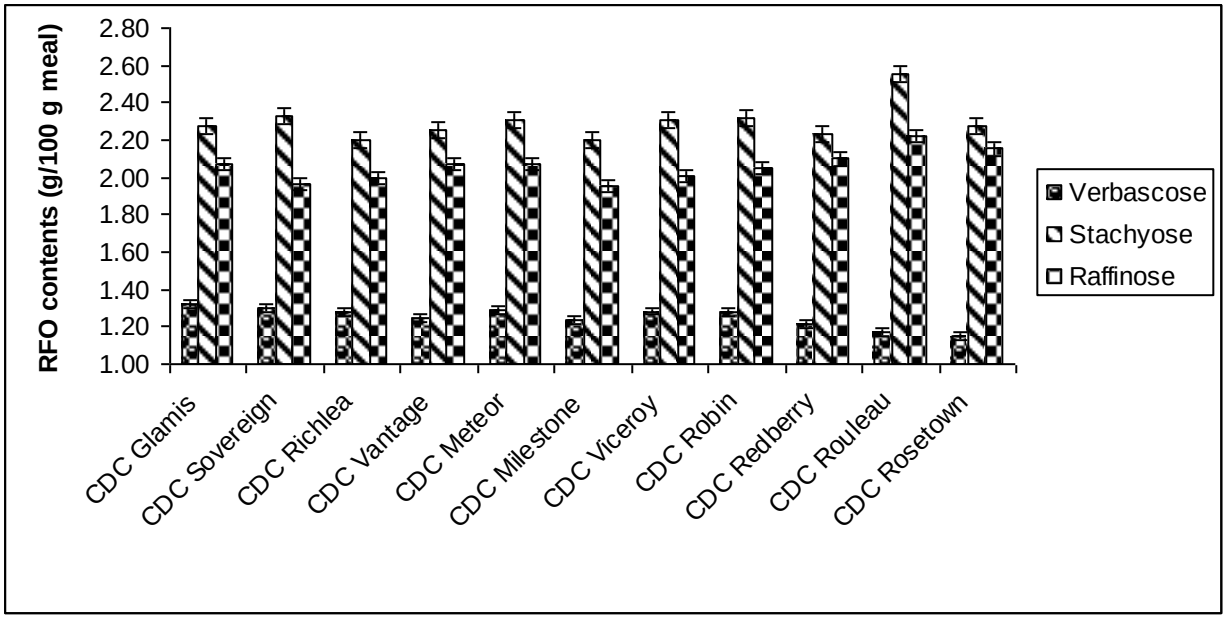

Figure 4.2. Verbascose, stachyose and raffinose concentration in seeds of selected lentil cultivars.

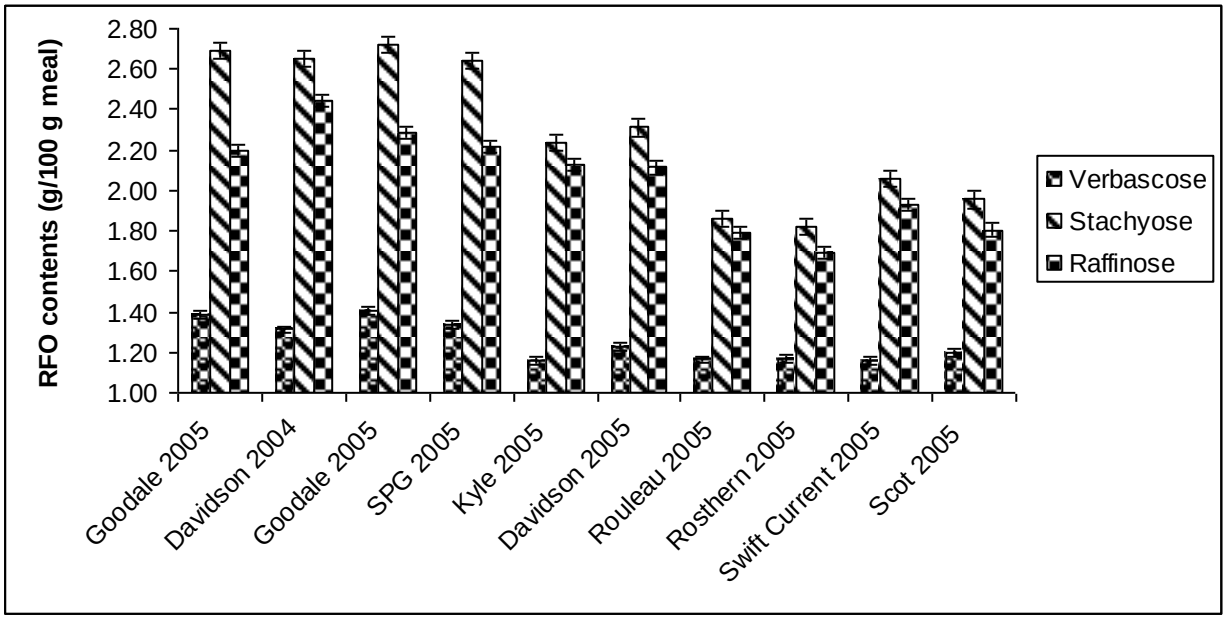

Figure 4.3. Verbascose, stachyose and raffinose concentration in different environments.

2005. Among these environments Goodale 2004 had the highest RFO concentration (5.4 mmoles 100 g^{-1}flour) followed by Rouleau 2005 (5.3 mmoles 100 g^{-1}flour), however, the RFO concentrations produced in these two environments were not significantly ($P \leq 0.05$) different.

The second group of environments with lower RFO concentrations included Kyle 2005 and Davidson 2004 and 2005 with the lowest mean RFO concentration of 4.6, 4.7 and 4.9 mmoles 100 g^{-1}flour, respectively.

The environments included in this study differed in total precipitation received during the growing season, soil type and temperature (Table 4.2). The significant differences in RFO concentrations in lentil cultivars grown in different environments may be attributed to these variables. Environments in the high RFO group received comparatively lower total precipitation during the lentil growing season than the low RFO group. Rouleau 2005 received 236.5 mm of precipitation which was the lowest of all environments; whereas Goodale 2004 received precipitation of 280 mm, though higher than the Swift Current 2005 and Davidson 2004 precipitation of 251.5 mm and 276.9 mm, respectively, was considerably lower than the precipitation received in other environments. Davidson 2005 and Kyle 2005 environments constituting the low RFO group received the highest precipitation of 393.7 mm and 382.2 mm respectively, of all the environments. These results show that lentil cultivars generally produced higher seed RFO in environments that received less precipitation compared to environments that received more precipitation. These results indicate that soil moisture concentration and resulting water stress influence the RFO concentration in lentil seeds. Similar trends were not observed between a specific soil zone and RFO concentration in lentil cultivars. All high RFO group environments except Swift Current 2005 fall in the Dark Brown soil zone, whereas three environments i.e. Kyle 2005 and Davidson 2004 and 2005, forming the low RFO group belonged to the Brown and Dark brown soil zones, respectively. The Brown soil zone is characterized by higher temperatures, low soil organic matter and low moisture compared to Dark Brown zone. No conclusive correlation between soil zone and RFO concentration was determined. Although no major study has previously been conducted on the effect of different environmental conditions on the RFO concentration in lentils, abiotic stresses such as temperature and drought have been reported to affect the concentrations of soluble sugars, including RFO, in seeds of other legume plants (Piotrowicz-Cieslak, 2006). The concentration and composition of RFO and sucrose in yellow lupin (*Lupinus luteus* L. cv. Juno) seeds varied with changes in temperature.

Table 4.3. Soluble carbohydrates (g 100 g^{-1}flour) of lentil cultivars across different environments in Saskatchewan.

Locations	Glucose (g 100 g^{-1}flour)	Sucrose (g 100 g^{-1}flour)	TRFO (mmoles 100 g^{-1}flour)
Goodale 04	0.07	1.65	5.4
Davidson 04	0.01	1.23	4.7
Goodale 05	0.03	1.48	5.1
SPG 05	0.03	1.55	5.1
Kyle 05	0.02	1.17	4.6
Davidson05	0.02	1.26	4.9
Rouleau 05	0.04	1.24	5.3
Rosthern 05	0.04	1.89	5.0
Swift Current 05	0.04	1.02	5.1
Scot 05	0.02	1.56	5.1
Mean	0.03	1.41	5.0
Range	0.01 - 0.07	1.02 - 1.89	4.6 - 5.4
SE of Means	0.01	0.02	0.01

Yellow lupin seeds that matured at high temperatures had more raffinose than seeds that matured at optimum temperatures and produced more stachyose. Seeds maturing at suboptimal temperatures also contained high concentrations of sucrose and myo-inositol (Piotrowicz-Cieslak, 2006). Similarly, drought (Piotrowicz-Cieskak et al 2007) and cold stress (Gilmour et al 2000) have been reported to affect RFO biosynthesis in plants.

The significant environmental effect on the RFO production in lentil seeds raises the question of the feasibility of a future lentil improvement program. Although the variations in the RFO concentration and other soluble carbohydrates in lentil cultivars due to differences in the soil and environmental conditions are statistically significant, they were not large and may not be significant with respect to the objective of lentil improvement for RFO concentration modification. The broad sense heritability (h^2) of RFO and sucrose calculated from the components of variance (Appendix 4.1) was 0.85 and 0.89, respectively. This suggests that selection of lentil cultivars and breeding lines with low RFO concentration might be possible. Correlation analyses showed a significant ($P \leq 0.01$) positive correlation between sucrose and RFO concentrations. This positive correlation between sucrose and RFO is expected as sucrose is a substrate for the RFO biosynthesis. These results are in agreement with a previous study (Karner et al 2004), where they reported a moderate correlation between sucrose and RFO production in pea genotypes. CDC Sovereign's highest sucrose and total RFO concentrations indicate this important relationship between RFO and sucrose concentration.

4.4.5 Summary and Conclusions

Soluble sugars of lentil cultivars varied significantly and were affected by different environmental conditions resulting in cross-over interaction. A strong positive correlation between sucrose and RFO concentration was observed. An inverse relationship between the total precipitation received during the lentil growing season at different growing environments and the RFO concentration of lentil cultivars was also observed. Changes in RFO concentration due to environmental effects, though statistically significant, were small from a RFO improvement perspective. High heritability of RFO would allow exploitation of natural variation in domesticated and wild genotypes for lentil quality improvement.

5 AN ASSESSMENT OF RAFFINOSE FAMILY OLIGOSACCHARIDES (RFO) AND SUCROSE CONCENTRATION IN GENUS *LENS*

5.1 Abstract

To determine the natural variation for sucrose and RFO concentration in lentils, we analyzed a subset of 168 *Lens* germplasm from the ICARDA collection. Significant ($P \leq 0.05$) variations were found for sucrose, total RFO concentrations and composition of the three main RFO (raffinose, stachyose and verbascose). Sucrose concentration in domesticated genotypes ranged from 0.5 - 2.7 g 100 g^{-1}flour and was overall higher than the concentrations observed in wild genotypes (0.1 - 1.7 g 100 g^{-1}flour). Similarly, the RFO concentrations in domesticated genotypes (4.0 - 6.1 mmoles 100 g^{-1}flour) were generally higher than in wild lentil genotypes (1.7 - 5.2 mmoles 100 g^{-1}flour). The verbascose, stachyose and raffinose concentrations in domesticated genotypes ranged from 1.1 - 1.6 g, 1.5 - 3.5 g, and 1.5 - 3.0 g, and were similar to concentrations measured in wild genotypes. *Lens ervoides* genotypes tended to have a higher verbascose and lower stachyose concentrations as compared to other genotypes. High raffinose and low verbascose concentrations were observed in *Lens nigricans* genotypes. The diversity in RFO, raffinose and verbascose concentrations was higher in wild genotypes as compared to domesticated lentils. Utilization of this natural variation in RFO concentration in genus *Lens* provides opportunities for improvement in lentil seed quality.

5.2 Introduction

Lentil (*Lens culinaris* Medik. ssp. *culinaris*), an annual self-pollinating grain legume, is the sixth most important pulse crop in the world after common bean, pea, chickpea, faba bean and cowpea in terms of production (FAO 2008). It originated in the Near East and is the oldest domesticated legume crop (Bahl et al 1993; Erskine 1997). Lentil plays an important role in increasing the health and fertility of soil due to its ability to fix atmospheric nitrogen. The protein, mineral and vitamin rich lentil seeds are a major source of human nutrition in many parts of the world. However, one of the major limitations to increased human lentil consumption is the high concentrations of raffinose family oligosaccharides (RFO) in the seeds.

RFO are soluble carbohydrates that are formed when D-galactose units are attached to the D-glucose moiety of a sucrose molecule through an alpha (1→6) linkage (Avigad and Dey 1997). The major RFO in lentil seed include stachyose, raffinose and verbascose. The total RFO concentration of lentils range from 1.8 - 7.5 g 100 g^{-1} dry matter with stachyose, raffinose and verbascose concentrations ranging from 1.1 - 4.0 g, 0.1 - 1.0 g and from non-detectable - 6.4 g 100 g^{-1}dry matter, respectively (Martínez-Villaluenga et al 2008). The lack of α-galactosidase enzyme in humans and monogastric animals results in an undigested passage of RFO from the stomach into the large intestine (Cristofaro et al 1974; Rackis 1975). RFO undergo anaerobic hydrolysis by microflora in the large intestine leading to production of carbon dioxide, hydrogen and methane gases which may cause abdominal discomfort, bloating and flatulence in humans (Cristofaro et al 1974; Price et al 1988; Kurbel et al 2006). These adverse effects restrict lentil consumption to low intake. To fully utilize the nutritional value of lentils, a reduction in RFO concentration in lentil seeds is desired. Several processing methods for reducing RFO concentration in pulses are available. Soaking (Jood et al 1985; Abdel-Gawad 1993; Somiari et al 1993; Vijayakumari et al 1996; Frias et al 2000), partial germination (Urbano et al 1995; Frias et al 1996), fermentation (Salunke et al 2000) and addition of α-galactosidase enzyme (Mansour and Khalil 1998) reduce RFO concentrations to varying degree, but the methods are time consuming, cause loss of energy and minerals, and lack consumer acceptability. Thus, development of low RFO cultivars is needed to improve lentil quality for human consumption.

The phylogeny of *Lens* (Cubero et al 2009) based on hybridization barriers, classifies *L. culinaris* ssp. *culinaris* and *L. c.* ssp. *orientalis* (Boiss.) Ponert into primary gene pool. The secondary gene pool includes *L. odemensis* (Ladiz.) and the tertiary gene pool includes *L. nigricans* (M. Bieb.) Godr. and *L. ervoides* (Brign.) Grande. The two remaining species, *L. tomentosus* (Ladiz.) and *L. lamottei* belong to either the secondary or tertiary gene pool. This classification agrees with morphological, randomly amplified polymorphic DNA and isozyme markers (Sarker and Erskine 2006). Land races and wild relatives of cultivated lentils show variation in several traits including disease resistance (Tullu et al 2010), drought tolerance (Hamdi and Erskine 1996), winter-hardiness (Hamdi et al 1996) and morphological traits (Robertson and Erskine 1997) and can provide a broad pool of potentially useful genetic resources for lentil seed quality improvement.

The International Centre for Agriculture in Dry Areas (ICARDA) has a global mandate for research on lentil improvement and holds the largest lentil germplasm collection in the world (Furman et al 2009). This collection of 10,509 genotypes is comprised of 8,789 genotypes of cultivated lentils from more than 70 different countries, 1,146 breeding lines and 574 genotypes of wild lentil species representing 23 countries (Furman 2006). Evaluation and screening of such a large germplasm collection for a specific trait is not practical, and therefore a core collections has been drawn from the germplasm collection. The core collection of 1,000 genotypes represents the genetic variability and diversity of the entire germplasm collection (Brown 1989) and was selected through hierarchical cluster analysis using agronomic traits and a two-step cluster analysis using agroclimatological data (Furman 2006; Furman et al 2009). Mini core collections consist of about 10% of the genotypes found in the core collection or 1% of the entire collection, but still represent the diversity of the entire core collection (Upadhyaya and Ortiz 2001). A mini core collection is lacking for lentils, but are available for several crops such as sorghum (Upadhyaya et al 2009), pigeon pea (Upadhyaya et al 2006b), peanut (Upadhyaya et al 2002), and chickpea (Upadhyaya and Ortiz 2001, Upadhyaya et al 2006a). The ICARDA germplasm collection has been used to assess variation in various traits of economic importance such as early maturity and drought stress (Ferguson and Robertson 1999) but has not been characterized for seed constituents. The objective of this study was to evaluate a subset of the ICARDA germplasm collection for variation in soluble sugars and RFO concentration with the long term objective to select lines for development of lentil cultivars with reduced RFO concentrations.

5.3 Materials and Methods

5.3.1 Plant material

In 2006 and 2007, an ICARDA lentil germplasm collection comprising 122 domesticated and 46 wild genotypes was grown at the University of Saskatchewan field plots and at the Saskatchewan Pulse Growers (SPG) farm, Saskatoon, Saskatchewan, Canada (Table 5.1). All domesticated lentil genotypes belonge to *Lens culinaris* (Medik) ssp. *culinaris* (Appendix 5.1). The wild lentil genotypes include 20 *Lens ervoides* (Brign.) Grande, 10 *Lens nigricans* (Bieb.) Godr., and three *Lens lamottei* Czefr. genotypes. The remaining 13 genotypes belong to *Lens*

odemensis Ladiz., *Lens culinaris* (Medik.) ssp. *orientalis* (Boiss.) Hand-Mazz. and *Lens tomentosus* Ladiz. (Appendix 5.2).

5.3.2 Determination of sucrose and total RFO concentrations

Harvested seeds were ground to flour using a cyclone sample mill (Udy Corporation, Fort Collins, Colorado, USA). The sucrose and total RFO concentrations of lentil flour (100 mg) were determined in duplicate samples using an enzyme-based method (McCleary et al 2006). Sucrose and RFO were sequentially hydrolyzed using α-galactosidase and invertase to glucose, followed by colorimetric determination of glucose using a raffinose/glucose assay kit (Megazyme International Ireland Ltd, Wicklow, Ireland) as described (Tahir et al 2011a).

5.3.3 Extraction of soluble carbohydrates and RFO composition analysis

Lentil seeds soluble carbohydrates were extracted with 80% (v/v) ethanol and separated by high performance size exclusion chromatography (HPLC-SEC) using a Sugar-Pak 1 column linked to a refractive index detector as reported (Tahir et al 2011a).

5.3.4 Shannon-Weaver diversity index (SDI)

The Shannon-Weaver Diversity Index (SDI) (Shannon and Weaver, 1949) for each trait in domesticated and wild genotypes was estimated according to Bhattacharjee et al (2007) as follows:

$$SDI = (-\Sigma ni=1\ Pi \times \log_e Pi)/\log_e n$$

Where, n= number of phenotypic classes, Pi = proportion of the total number of entries in the i^{th} class.

Similarly, SDI for different geographical regions was estimated by grouping domesticated genotypes into six regions based on the geographical proximity of the county of origin of domesticated genotypes (Table 5.2). Minitab software version 16 (Minitab Inc., State College, PA, USA) was used to group the data into classes. The number of genotypes in each class was determined and the SDI was calculated using the formula described above.

5.3.5 Statistical analyses

Analyses of variance (ANOVA) and correlation analyses were conducted using SAS version 9 (SAS Institute Inc., Cary, NC, USA).

Table 5.1. Field locations used in study.

Site	Location	Soil Type	Soil Zone	Coordinates
SPG Farm	10 km south east of Saskatoon	Clay loam	Dark Brown	52° 04' 0" N. 106° 28' 0" W.
University of Saskatchewan	Saskatoon	Clay	Dark Brown	52° 7′ 0″ N. 106° 39′ 0″ W.

Table 5.2. Shannon-Weaver diversity index (SDI) estimates for domesticated genotypes.

Geographical regions	**Number of genotypes**	**SDI for sucrose**	**SDI for total RFO**
1. Afghanistan, Iran, Tajikistan, Uzbekistan, Russia, China	19	0.59	0.55
2. India, Pakistan, Bangladesh, Nepal	27	0.35	0.55
3. Turkey, Syria, Jordon, Palestine, Lebanon, Iraq, Azerbaijan, Armenia	23	0.71	0.42
4. Europe (Bulgaria, Cyprus, Croatia, Czech Republic, France, Germany Greece, Hungary, Italy, Macedonia, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, United Kingdom, Yugoslavia (former)	21	0.47	0.45
5. Egypt, Ethiopia, Sudan, Saudi Arabia, Yemen, Tunisia, Morocco, Libya, Algeria	17	0.48	0.50
6. Americas (Argentina, Brazil, Chile, Colombia, Uruguay, Guatemala, Canada, United States, Mexico)	10	0.50	0.51

5.4 Results and Discussion

5.4.1 Total RFO and sucrose concentration and composition of domesticated lentils

The *Lens culinaris* (Medik.) ssp. *culinaris* genotypes included in this study originated from 58 different countries. The majority of genotypes originated from India (16%), Iran (8%), Syria (6%), Turkey (5%), Ethiopia (4%), Jordan (4%), and Afghanistan (3%). Analysis of soluble carbohydrates of domesticated lentils revealed significant variation ($P \leq 0.001$) in sucrose and total RFO concentrations (Table 5.3). The sucrose concentrations ranged from 0.5 - 2.7 g 100 g^{-1}flour (Fig. 5.1, Appendix 5.1) and were similar to sucrose concentration of 1.1 - 3.0 g 100 g^{-1}dry matter reported for Canadian and Spanish lentil genotypes (Vidal-Valverde et al. 1993a, 1993b; Wang and Daun, 2006). Duncan's multiple range test showed that genotype ILL 927 from Kastamonu, Turkey had the highest sucrose concentration which was significantly ($P \leq 0.05$) different from sucrose concentrations of all other genotypes. The lowest sucrose concentrations were recorded for genotypes ILL 4915 from Croatia and ILL 4778 from Uruguay.

The total RFO concentration of domesticated genotypes ranged from 4.0 - 6.1 mmoles 100 g^{-1}flour (Fig. 5.2, Appendix 5.1) and the values range was similar to values previously reported for lentils (Tahir et al 2011a). The highest total RFO concentration was observed in genotype ILL 1220 from Fars, Iran and the lowest total RFO concentration was recorded for genotype ILL 4778 which originated in Uruguay. Interestingly, genotype ILL 4778 also had the lowest sucrose concentration. However, the positive relationship between total RFO and sucrose concentration was not significant ($P \leq 0.05$) for the whole population analyzed. No obvious trend could be observed in the total RFO concentrations of domesticated genotypes based on the geographical origin (Appendix 5.1).

Composition analysis of RFO indicated significant differences ($P \leq 0.05$) in the individual RFO concentrations of domesticated genotypes. Verbascose, stachyose and raffinose were present in seeds of all domesticated genotypes and their concentrations ranged from 1.1 - 1.6 g, 1.5 - 3.5 g, and 1.5 - 3.0 g 100 g^{-1}flour, respectively (Fig. 5.3, Appendix 5.1). The values of individual concentrations of verbascose, stachyose and raffinose matched closely with values

Table 5.3. Analysis of variance of soluble carbohydrates content and composition of domesticated lentil genotypes.

		Mean Square				
Source	DF	Total RFO	Sucrose	Verbascose	Stachyose	Raffinose
Environments	1	7.06*	4.04*	0.18*	0.26^{ns}	0.25^{ns}
Genotypes	121	0.48*	0.40*	0.04*	0.40*	0.22*
Environment × Genotype	121	0.13^{ns}	0.04^{ns}	0.00^{ns}	0.10^{ns}	0.08^{ns}
Replication	1	0.31	6.30	0.30	1.35	4.37
Residual	191	0.14	0.05	0.01	0.12	0.08
CV		7.42	18.29	7.38	13.30	12.30
R-square		0.76	0.88	0.72	0.68	0.70

* and ns=significant at $P \leq 0.01$ and not significant, respectively.

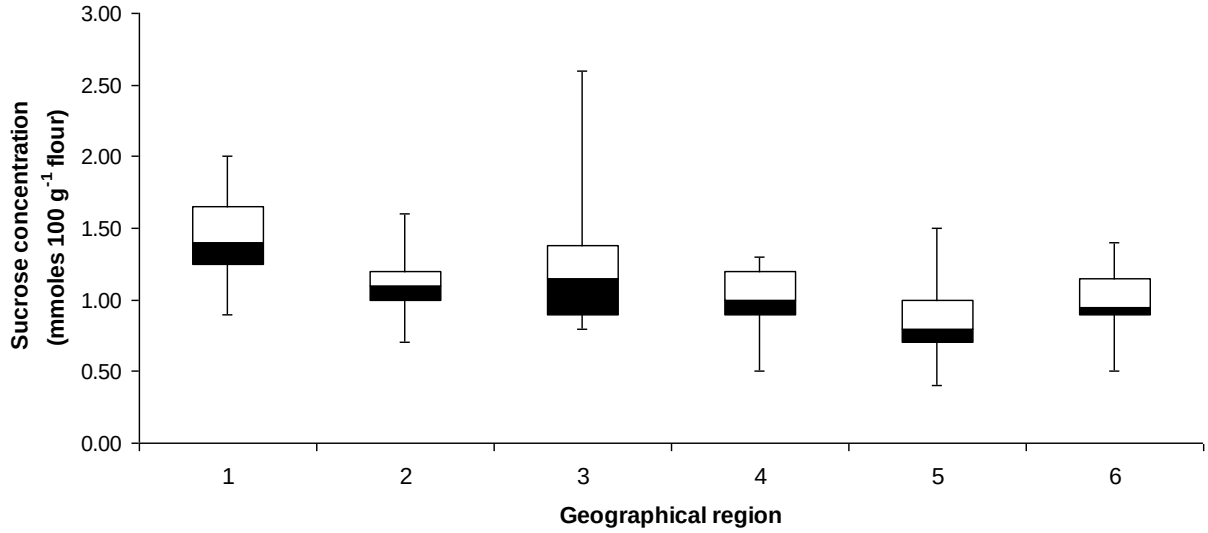

Figure 5.1. Sucrose concentration of domesticated lentils from various geographical regions. Countries in the regions 1 to 6 are presented in Table 5.2.
White box of graph represents the 75th percentile and black area of box the 50th percentile of sucrose concentration. The lower and upper error bars on the boxes indicate range between first quartile and minimum sucrose concentration and range between maximum sucrose concentration and third quartile, respectively.

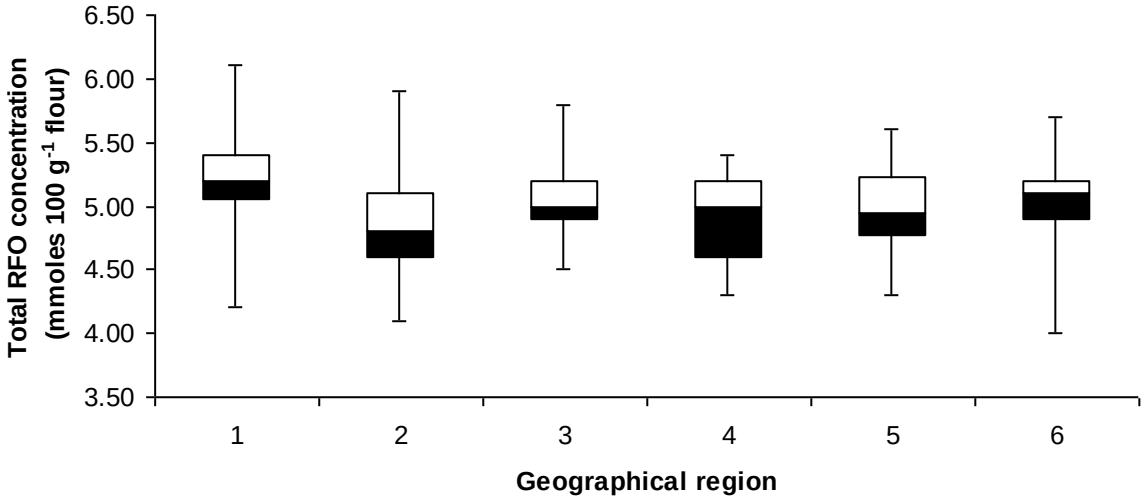

Figure 5.2. Total RFO concentration of domesticated lentil genotypes from various geographical regions. Countries in the regions 1 to 6 are presented in Table 5.2.
White box represents the 75th percentile and black area of box the 50th percentile of total RFO concentration. The lower and upper error bars on the boxes indicate range between first quartile and minimum total RFO concentration and range between maximum total RFO concentration and third quartile, respectively.

reported for lentil genotypes grown in Canada, Egypt, Spain and other countries (Wang and Daun 2006; El-Adawy et al 2003; Frias et al 1994, 1995, 1996; Vidal-Valverde et al 1993a, 1993b). Variations in the relative concentrations of stachyose and raffinose among domesticated lentil genotypes were observed. Stachyose was the predominant RFO in domesticated genotypes. In three genotypes, ILL 5424, ILL 5425 and ILL 7089 raffinose concentrations was higher than stachyose concentration, however, these differences were not significant ($P \leq 0.05$) (Appendix 5.1). In genotypes, ILL 7791, ILL 6853, ILL 3487, ILL 6378, ILL 3597, ILL 5425 and ILL 6967, the concentrations of stachyose and raffinose were similiar.

5.4.2 Total RFO and sucrose concentration and composition in wild lentils

Wild lentil genotypes showed significant ($P \leq 0.05$) differences in sucrose and total RFO concentrations (Table 5.4). The sucrose concentrations ranged from 0.1 - 1.7 g 100 g^{-1}flour (Fig. 5.4, Appendix 5.2) and were overall lower than the sucrose concentrations observed in domesticated lentil genotypes (Fig 5.1, Appendix 5.1). As seed size positivly correlates with high sucrose concentration in lentils (Tahir et al 2011b), the higher sucrose concentration in domesticated genotypes may be due to selection for large seeds throughout the process of domestication. Alternatively, selection for sweetness may have increased the sucrose concentration in domesticated lentils. The low sucrose concentration in wild species may also be associated with their lower total RFO concentration, since sucrose and RFO concentrations often correlate in lentil and pea genotypes (Karner et al 2004; Tahir et al 2011b). Duncan's multiple range test showed that sucrose concentrations of *Lens odemensis* genotype ILWL 202 originating from Turkey and *Lens culinaris* ssp. *orientalis* genotype ILWL 73 originating from Cyprus were significantly higher ($P \leq 0.05$) than all other wild lentil genotypes (Appendix.5.2). The lowest sucrose concentration was found in *Lens ervoides* genotype ILWL 42 from Italy, however, sucrose concentration of this genotype was not significantly different than several other *Lens ervoides* genotypes. In the primary and secondary gene pool of the cultivated lentil, i.e. *L. c.* ssp, *orientalis, L. odemensis* and *L. tomentosus*, the sucrose concentrations tended to be higher than in the tertiary gene pool (Fig. 5.4).

The total RFO concentration of wild lentils varied from 1.7 - 5.2 mmoles 100 g^{-1} flour (Fig. 5.5, Appendix 5.2). The lowest and highest total RFO concentrations were observed in *L. ervoides* genotype ILWL 285 and *L. odemensis* genotype ILWL 20, respectively. The variation

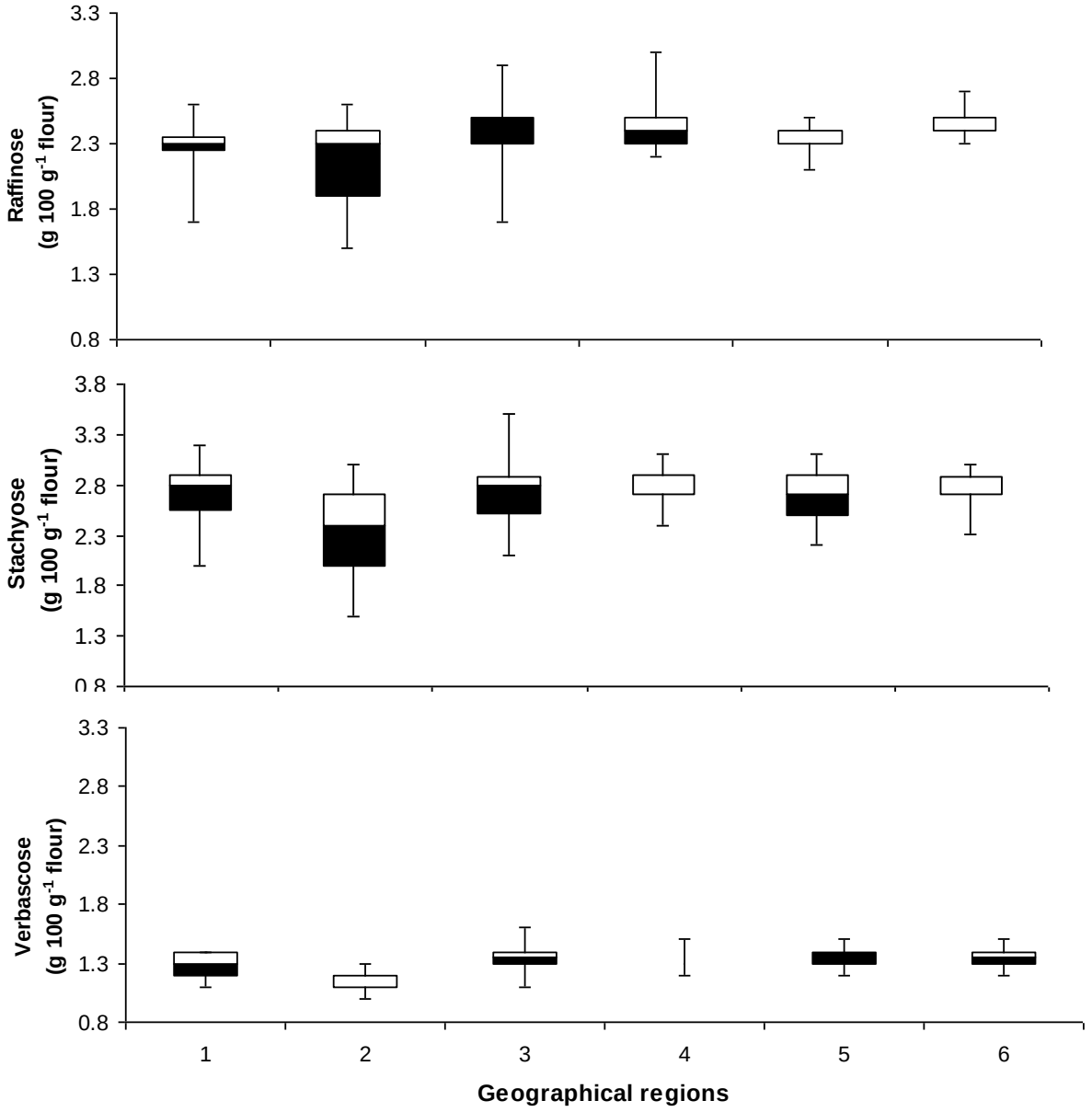

Figure 5.3. RFO composition of domesticated lentil genotypes. Range of raffinose, stachyose and verbascose concentrations are shown. Countries in the regions 1 to 6 are presented in Table 5.2. White box represents the 75th percentile and black area of box the 50th percentile. The lower and upper error bars on the boxes indicate range between first quartile and minimum individual oligosaccharides concentration and range between maximum oligosaccharide concentration and third quartile, respectively.

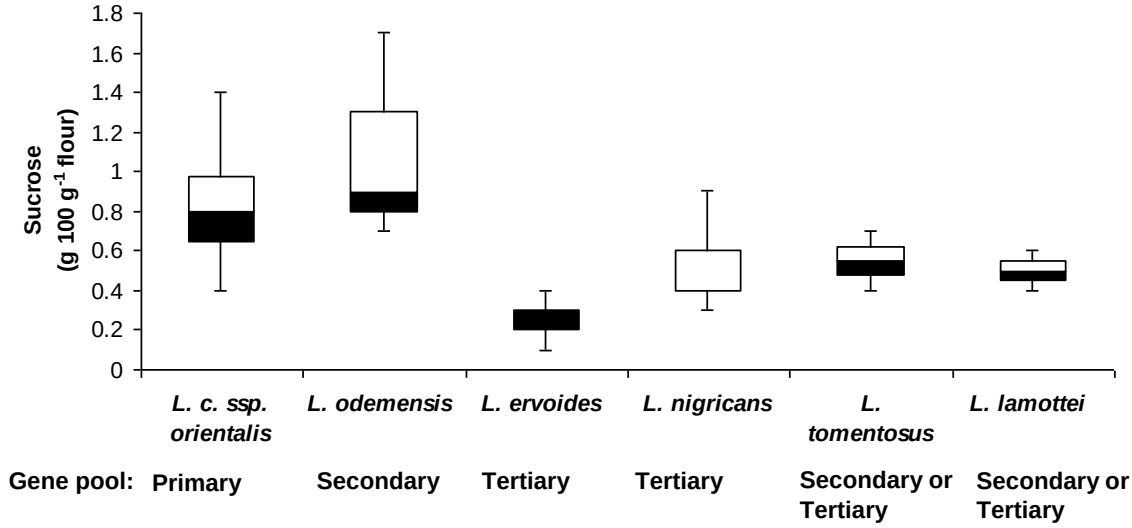

Figure 5.4. Sucrose concentration in seeds of wild lentil genotypes.
White box represents the 75th percentile and black area of the box the 50th percentile of the sucrose concentration. The lower and upper error bars on the boxes indicate range between first quartile and minimum sucrose concentration and range between maximum sucrose concentration and third quartile, respectively.

Table 5.4. Analysis of variance of soluble carbohydrates content and composition in wild lentil genotypes.

Source	DF	Mean Square				
		Total RFO	Sucrose	Verbascose	Stachyose	Raffinose
Environments	1	0.55^{ns}	0.09 ^{ns}	0.09^{ns}	1.35*	1.35*
Genotypes	44	1.86*	0.27*	0.27*	0.17*	0.28*
Environment × Genotype	36	0.33^{ns}	0.10*	0.08*	0.14*	0.14*
Replication	1	0.84^{ns}	0.05^{ns}	0.00^{ns}	0.21^{ns}	0.01^{ns}
Residual		0.28	0.03	0.04	0.04	0.08
Residual DF		46	46	53	57	57
Corrected Total		128	129	130	134	134
CV		17.28	34.82	15.05	9.46	11.49
R-square		0.89	0.92	0.87	0.86	0.80

*, ns=significant at $P \leq 0.05$ and not significant, respectively.

in total RFO concentration in wild genotypes was higher than observed in domesticated genotypes (Fig. 5.2) and previous reports for lentil cultivars (Tahir et al 2011a). The primary and secondary gene pool species, (*L. c.* ssp, *orientalis* and *L. odemensis*) showed overall higher total RFO concentration than tertiary gene pool species (*L. ervoides*, *L.* lamottei and *L. nigricans*) (Fig. 5.5).

Composition analysis of RFO showed significant ($P \leq 0.05$) variation for verbascose, stachyose and raffinose concentrations in wild lentil genotypes (Table 5.4, Fig. 5.6, Appendix 5.2). The verbascose concentration varied from 0.3 - 1.7 g 100 g^{-1}flour and the highest value was observed in *L. ervoides* genotype ILWL 0 originating in Yugoslavia. *L. nigricans* genotype ILWL 327 from Ukraine showed the lowest verbascose concentration. Variation in verbascose concentration was very low among the primary and secondary gene pool species (*L. c.* ssp. *orientalis* and *L. odemensis*) whereas *L. ervoides* genotypes showed a relatively high variability in verbascose concentrations.

The stachyose concentration of wild genotypes varied significantly ($P \leq 0.05$) and ranged from 1.5 - 2.6 g 100 g^{-1}flour. *Lens culinaris* ssp. *orientalis* genotypes ILWL 70 from Iran and ILWL 380 from Turkmenistan had the highest stachyose concentrations, whereas *L. ervoides* genotype ILWL 133 from Syria had the lowest stachyose concentration. The largest variation in stachyose concentration (1.7 - 2.6 g 100 g^{-1}flour) was noted among genotypes of the primary gene pool i.e. *L. c.* ssp. *orientalis* (Fig.5.6). Species belonging to *L. ervoides* and *L. lamottei* had relatively lower stachyose concentrations as compared to other wild lentil genotypes (Fig. 5.6).

The raffinose concentrations of wild lentil species also varied significantly ($P \leq 0.05$) and ranged from 1.5 - 3.0 g 100 g^{-1}flour. The highest raffinose concentration was observed in *L. nigricans* genotype ILWL 327 originating from Ukraine whereas the lowest concentration was found in *L. ervoides* genotype ILL 133 from Syria. Interestingly, genotype ILWL 327 also had the lowest verbascose concentration. Genotypes of *L. ervoides* generally showed lower raffinose concentrations than the other gene pools.

5.4.3 Shannon-Weaver diversity index (SDI)

The Shannon-Weaver diversity index (SDI) (Shannon and Weaver, 1949) was used as a measure of phenotypic diversity of total RFO and sucrose concentration in domesticated and

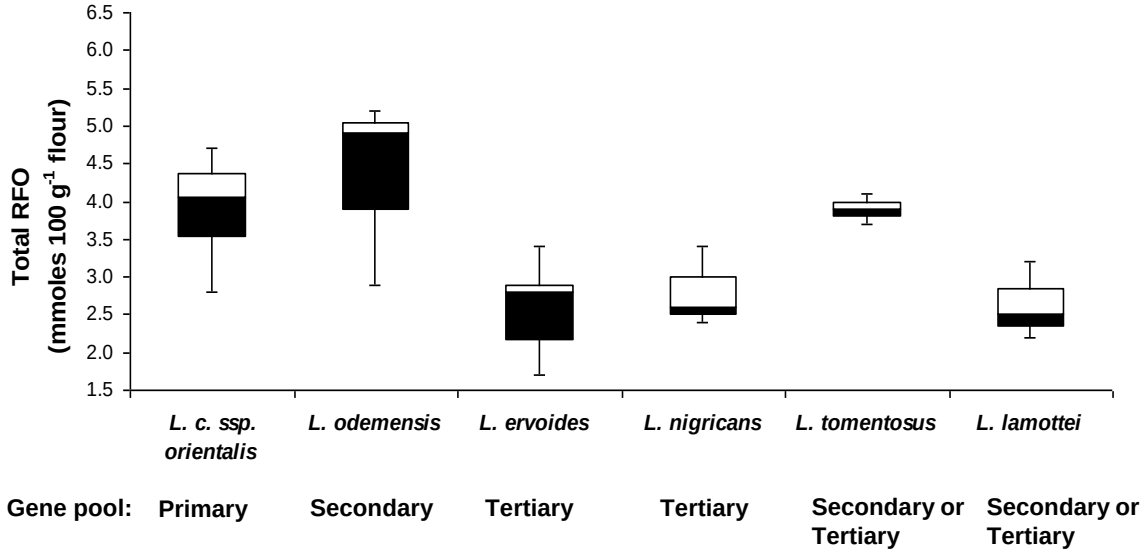

Figure 5.5. Total RFO concentration in seeds of wild species and subspecies of genus Lens. White box represents the 75th percentile and black area of the box the 50th percentile of total RFO concentration. The lower and upper error bars on the boxes indicate range between first quartile and minimum total RFO concentration and range between maximum total RFO concentration and third quartile, respectively.

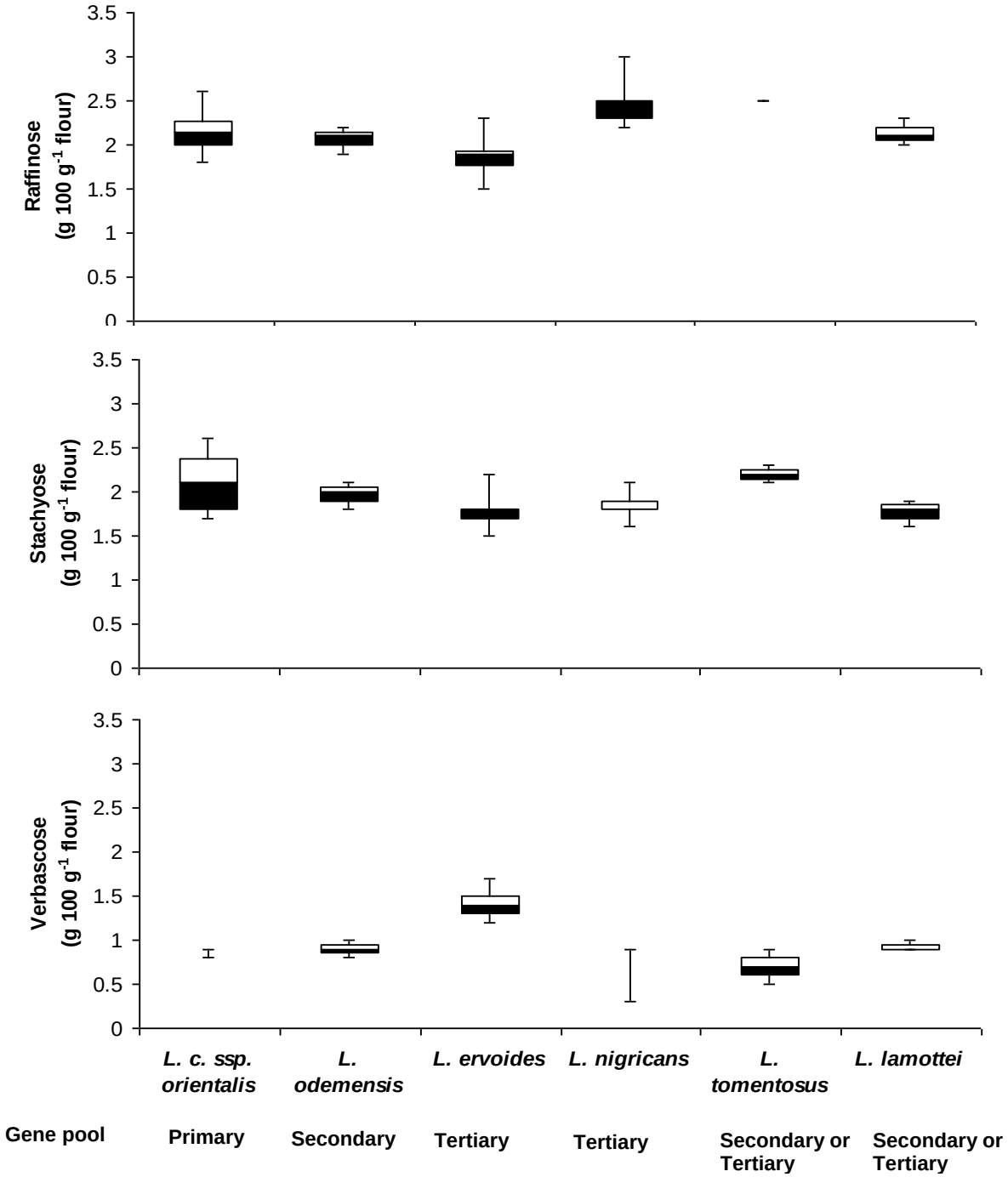

Figure 5.6. RFO in seeds of wild lentil genotypes. Ranges of raffinose, stachyose and verbascose concentrations are shown.
White box represents the 75th percentile and black area of the box the 50th percentile of individual oligosaccharides. The lower and upper error bars on the boxes indicate range between first quartile and minimum oligosaccharide concentration and range between maximum oligosaccharide concentration and third quartile, respectively.

wild genotypes and for assessing sucrose and total RFO diversity among different geographical regions (Table 5.2, Table 5.5). A higher Shannon-Weaver diversity index was estimated for total RFO concentration and composition of individual oligosaccharides of the raffinose family in wild genotypes (SDI = 0.79) compared to domesticated genotypes (SDI = 0.54) (Table 5.1). Thus, the total RFO trait is genetically more diverse in wild lentil species and subspecies than cultvated species (Table 5.5). Shannon-Weaver diversity index for sucrose concentration, however, was lower in wild genotypes (SDI = 0.46) compared to domesticated genotype (SDI = 0.60) indicating low genetic diversity for sucrose concentration in wild genotypes (Table 5.5).

In relation to geographical regions, the highest and lowest diversity in sucrose concentration was observed in third region (SDI = 0.71) and second (SDI = 0.35), respectively (Table 5.2). Region three included Turkey, Syria, Jordon, Palestine, Lebanon, Iraq, Azerbaijan and Armenia whereas region two represents India, Pakistan, Bangladesh and Nepal. It may be mentioned that region three include Turkey and Syria which are regarded as the center of origin of lentil (Erskine 1997). Similarly, the highest diversity in total RFO concentration (SDI = 0.55) was observed in both regions one and two representing countries of the Central Asia and South Asia, respectively (Fig. 5.2) which includes countries with the largest consumers and producers of lentil. The lowest diversity in total RFO concentration (SDI = 0.42) was observed in region three comprised of Turkey, Syria, Jordon, Palestine, Lebanon, Iraq, Azerbaijan and Armenia.

5.4.4 Variation in RFO and its use in lentil seed quality improvement

Large differences in the RFO concentrations and profiles were observed in both domesticated and wild lentil genotypes. A higher variation in total RFO concentration was found in wild species and subspecies of lentils (1.7 - 5.2 mmoles 100 g^{-1}flour) compared to RFO concentration observed in domesticated (*L. c.* ssp. *culinaris*) genotypes (4.0 - 6.1 mmoles 100 g^{-1}flour). *Lens ervoides* genotypes ILWL 285, ILWL 328, ILWL 412, ILWL 42 and some other wild genotypes with RFO concentrations of almost one-half compared to the total RFO concentrations of some lentil cultivars reported previously (Tahir et al 2011a). These genotypes may provide useful genes for low RFO trait introgression and development of lentil cultivars with low RFO concentrations. Similarly, wild lentils with variation in individual oligosaccharides may be used to study the activities of enzymes of RFO biosynthetic pathway and RFO accumulation in lentil seeds. Although variations in RFO concentration and

composition for such a large collection of different species and subspecies have not been reported previously, useful variation in other important traits such as disease resistance and agronomic traits are known (Gupta and Sharma 2006; Tullu et al 2010). The use of wild germplasm in breeding is often limited by difficulties with interspecific crossing and linkage drag of undesirable agronomic traits. The different species of the genus *Lens* are classified into *culinaris, nigricans* and *tomentosus* groups based on their crossability (Hancock 2004). The *culinaris* group includes the cultivated lentil *Lens c.* ssp. *culinaris*, ssp. *orientalis*, and *Lens. odemensis.* These subspecies are crossing compatible and crosses result in fully or partially fertile F_1 progenies. The nigricans group is comprised of the intercrossable species of *Lens nigricans, Lens lamotte* and *Lens ervoides,* whereas *Lens tomentosus* constitute the *tomentosus* group. Crosses between different groups leads to embryo abortion (Ladizinsky et al 1984; Van Oss et al 1997). Hybrids of *L. culinaris, L. orientalis* and *L. odemensis* with *L. nigricans* do not produce viable seed (Ladizinsky et al 1984, 1985). However, hybrids of *L. culinaris* with *L. ervoides* can produce viable seed with the use of embryo rescue techniques (Ladizinsky et al 1985). Based on interspecific hybridization the wild progenitor of the cultigens, *L. c.* ssp. *orientalis* is classified in the primary gene pool whereas *L. odemensis* and *L. tomentosus* in the secondary gene pool (Sharma and Chahota 2004; Cubero et al 2009). Similarly, *L. nigricans, Lens ervoides and L. lamotte* have been grouped in the tertiary gene pool. *L. ervoides* can hybridize with cultivated lentils using embryo rescue techniques and thus can become a member of secondary gene pool (Ladizinsky 1993; Sharma and Chahota 2004). Introgression of low RFO traits from the secondary and tertiary gene pools into cultivated lentils will, therefore, require the use of embryo rescue techniques. Alternatively, lentil seed quality can be improved by producing low RFO mutant lines of cultivated lentils and subsequently using them in low RFO cultivar development.

5.4.5 Summary and Conclusions

We evaluated the genetic variation for total RFO and sucrose concentration and composition of individual oligosaccharides in 122 domesticated lentil genotypes originating from 56 different countries and 46 wild lentil genotypes from 21 countries representing the genetic resources of the wild species available in the primary, secondary and tertiary gene pools of the genus *Lens.* Results revealed significant variation in RFO and sucrose concentration and in composition of

Table 5.5. Shannon-Weaver diversity index (SDI) estimates of soluble carbohydrates in domesticated and wild lentil genotypes.

Trait	Shannon-Weaver Diversity Index	
	Domesticated	Wild
Sucrose	0.60	0.46
Total RFO	0.54	0.79
Raffinose	0.50	0.71
Stachyose	0.64	0.48
Verbascose	0.34	0.51

individual oligosaccharides in both domesticated and wild lentil genotypes. *Lens ervoides* genotype ILWL 285 and *Lens culinaris* ssp. *culinaris* genotype ILL 4778 had the lowest total RFO concentrations in the wild and domesticated genotypes, respectively. A non- significant positive correlation existed between sucrose and RFO concentration, however, no apparent trend in RFO concentration was observed based on geographical origin of the genotypes. A higher variation in RFO composition was observed in wild lentil genotypes as compared to domesticated genotypes. Similarly, more genetic diversity in total RFO and composition was observed in wild genotypes as compared to domesticated genotypes. Overall, higher verbascose and lower stachyose concentrations were observed in *Lens ervoides* genotypes, whereas higher raffinose and lower verbascose concentrations were observed in *Lens nigricans* genotypes. Genotypes with contrasting RFO concentrations and compositions may be used for the development of low RFO cultivars and for understanding the RFO biosynthesis in lentils in the future.

6 RELATIONSHIP BETWEEN GALACTINOL SYNTHASE ACTIVITY AND ACCUMULATION OF RFO IN LENTIL SEEDS

6.1 Abstract

Galactinol synthase (GS; EC 2.4.1.123) catalyzes the reaction between UDP-galactose and myo-inositol to synthesize galactinol, a galactosyl donor for raffinose family oligosaccharide (RFO) biosynthesis. To determine whether GS activity and RFO concentration relate, we studied GS enzyme activity in the crude cell extracts of developing lentil seeds of genotypes with varying RFO concentrations. Sucrose, galactinol and myo-inositol concentrations were also analyzed at different stages of seed development. GS activity was highest in the initial phases of seed development with a peak at 16 DAF but gradually reduced as the seeds matured. The galactinol concentration peaked at the middle stage of seed development and thereafter declined. Genotypes with the highest GS activity produced 113 and 104 nmole UDP min^{-1} mg^{-1} protein, whereas genotypes with the lowest galactinol synthase activity had values of 22 and 62 nmole UDP min^{-1} mg^{-1} protein. The level of galactinol synthase activity, however, did not show any particular trend based on the differences in total RFO concentration of lentil genotypes. Sucrose and galactinol concentration of different genotypes ranged from 1.4 - 4.1 g and 0.16 - 0.44 g 100 g^{-1}, respectively, during different stages of seed development. No association between galactinol and RFO concentration was observed between different lentil genotypes. These results may suggest a non-regulatory role of galactinol synthase in RFO biosynthesis and accumulation in lentil seeds.

6.2 Introduction

Sucrose and α-galactosides of the raffinose family oligosaccharides (RFO) are the predominant soluble carbohydrates in lentil seeds. RFO accumulate in seeds, roots, and tubers of plants (Dey 1980; Andersen et al 2005) and have protective physiological functions during desiccation and cold exposure (Clegg et al 1982; Obendorf et al 1998). Lentil seeds also contain galactosyl cyclitols e.g. ciceritol, but at much lower concentration than RFO. RFO and galactosyl cyclitols are synthesized by transfer of galactosyl residues from a galactosyl donor to an acceptor by specific transferases. Both types of galactosides share common α-(1→6)-O-D linkages

between galactose moieties (Lahuta et al 2010). For α-galactosides biosynthesis the galactosyl donor can be galactinol, UDP-D-galactose, or RFO themselves, whereas galactosyl acceptors can be sucrose, RFO or cyclitols such as ononitol and pinitol (Lehle and Tanner 1972; Hoch et al 1999; Peterbauer and Richter 2001). UDP-D-galactose, however, is not used directly for RFO synthesis, but instead its galactosyl residue is transferred to *myo*-inositol by galactinol synthase to yield galactinol which is a specific galactosyl donor for RFO biosynthesis (Peterbauer and Richter 2001). Attempts to understand the RFO biosynthesis and accumulation have focused on a possible regulatory role of galactinol synthase as well as galactose acceptors in RFO biosynthetic pathway in plants. A mutant soybean (*Glycine max*), with drastically reduced *myo*-inositol concentration produces only low levels of both galactinol and RFO concentrations in seeds (Hitz et al 2002); thus indicating a role for the initial substrates on RFO accumulation. Both pea and barley seeds, show a significant correlation between *myo*-inositol and RFO concentrations. Similarly, a strong relationship exists between sucrose and RFO concentration as well as between *myo*-inositol and galactinol concentration (Karner et al 2004).

Different forms of plant GS catalyze the formation of several cyclitols in addition to the formation of galactinol from myo-inositol and UDP-galactose. GS from soybean seeds (Obendorf et al 2004); buckwheat (Ueda et al 2005) and hairy vetch (Lahuta et al 2005a) synthesize fagopyritols from UDP-galactose and D-chiro-inositol, but cannot synthesize isomers of galactosyl pinitol. Similarly, different forms of GS identified in *Arabidopsis thaliana* (Taji et al 2002) and *Ajuga reptans* (Sprenger and Keller 2000). However, no isoforms of GS or their function has been reported in lentils. Stachyose synthase and raffinose synthase are believed to participate in the formation of isomers of galactosyl pinitol (Peterbauer and Richter 2001), ciceritol (Hoch et al 1999) and possibly other di- and tri-galactosyl cyclitols. This raises the possibility that both galactosyl cyclitols and RFO share a common biosynthetic pathway and that the accumulation of RFO or galactosyl cyclitols may be regulated by the concentration of galactose acceptors (sucrose for RFOs and cyclitols for galactosyl cyclitols). However, Lahuta et al (2010) reported that feeding fine-leaved vetch raceme explants, which would normally accumulate both cyclitols and RFO, with myo-inositol or sucrose promoted accumulation of galactosyl pinitol only, thus negating the regulatory role of galactosyl acceptors in RFO biosynthesis.

Several studies have been conducted to find a relationship between GS activity and RFO accumulation in plants. A significant positive correlation (r = 0.84) between GS activity and proportion of total sugars as raffinose oligosaccharides, and a significant negative correlation (r = -0.73) for GS activity and sucrose concentration was found in a survey of 20 different plant species (Handley et al 1983). Similarly, in four soybean genotypes with contrasting RFO concentrations, the GS activity was higher in high-RFO accumulating genotypes as compared to low-RFO genotypes (Saravitz et al 1987). Furthermore, differences in total amount of RFO deposited during seed development can be correlated with GS activity in soybean (Lowell and Kuo 1989). *Cucumis sativus* leaves also show a positive correlation between GS activity and accumulation of RFO at different developmental stages (Pharr and Sox 1984). Transgenic *Arabidopsis thaliana* plants overexpressing a GS isoform show increased galactinol and raffinose concentrations (Taji et al 2002), indicating that GS may be an important regulator of carbon partitioning between sucrose and RFO in plant leaves and seeds. However, a role of GS in RFO accumulation has been contradicted by several reports. In a study of two pea cultivars with contrasting RFO composition, the differences in GS activity were insignificant and GS activity was actually 50-to 150-fold higher than required for the observed RFO accumulation in seeds (Peterbauer et al 2001). Furthermore, neither GS enzyme activity nor galactinol or raffinose quantities can be related to maize GS transcript abundance during seed development (Zhao et al 2004). Instead, galactinol concentration is closely related to myo-inositol quantities during maize development and early germination (Zhao et al 2004). Similarly, developing and germinating tomato seeds do not show correlation between GS mRNA accumulation and raffinose concentration (Downie et al 2003). In pea seeds, the myo-inositol concentration controls biosynthesis of galactinol rather than GS and elevated galactinol concentration is not always correlated with high RFO accumulation (Karner et al 2004). Together, these findings show a lack of consensus between GS activity and RFO accumulation and do not conclusively support a regulatory role of GS in carbon partitioning between sucrose and RFO in plants.

Lentil accumulate high quantities of RFO, however, the individual enzymes involved in RFO biosynthesis have not been studied in lentils. The objective of this study was to analyze GS activity during lentil seed development and to determine how it relates to RFO concentrations in mature seeds.

6.3 Material and Methods

6.3.1 Materials

Six domesticated lentil genotypes with varying RFO concentrations selected from the ICARDA lentil germplasm collection and CDC Redberry were grown in two gallon pots in a growth chamber under 21 °C day and 15 °C night temperatures (Table 6.1). The photoperiod was set at 18 hr light (250 µmol m^2 s^{-1}) and 6 hr dark. Slow release fertilizer (Nutricote 14-14-14) was applied a week after emergence. Developing lentil seeds were collected at 16, 24, 32 and 40 days after flowering (DAF) and immediately frozen in liquid nitrogen and stored at -80 °C until use (Appendix 6.1). Seeds at 40 DAF for genotype ILL4778 could not be collected due to disease and insect infestation.

6.3.2 Preparation of seed crude extract

Galactinol synthase activity in cell crude extracts was determined in four replicates following the method of Ribeiro et al (2000) with some modifications. Briefly, developing lentil seeds (0.3 g) were ground in liquid nitrogen using pestle and mortar and extracted with 0.3 mL ice-cold extraction buffer (50 mM HEPES, pH 7.5, 1 mM DTT and 1 mM PMSF). The homogenate was centrifuged at 27,000 x *g* for 30 min at 4 °C. The total protein concentration of the extract was determined by a dye binding assay (Bradford 1976) using a standard curve developed with different bovine serum albumin (BSA) concentrations (Appendix 6.2b).

6.3.2.1 GS assay

The GS assay reaction mixture (100 µL) contained crude cell extract (50 µL) with requisite protein concentration, 60 mM *myo*-inositol, 2 mM DTT, 50 mM HEPES buffer (pH 7.5), 4 mM $MnCl_2$, 20 µg of BSA and 4 mM UDP-gal. A control reaction mixture contained 50 µL heat-killed extract obtained by boiling for 2 min. The reaction mixture was incubated at 32 ºC for 30 min and terminated by boiling for 2 min. The terminated reaction was diluted with 500 µL water, and combined with 0.3 U of insect or potato apyrase solution in 10 µL, and 150 µL of apyrase reaction mixture (250 mM Tris-HCl buffer, pH 7.5, 25 mM KCl, 7.5 mM $CaCl_2$, 0.5 mM Na-EDTA, and 50 mM glucose). The reactions were incubated for 10 min at 37 °C and stopped by adding 60 µL of 75% v/v TCA. The tubes were cooled on ice for 10 min and centrifuged at 3,000 x *g* for 10 min.

Table 6.1. Mean soluble carbohydrates concentration and RFO composition of selected lentil genotypes.

Genotype	Country of origin	Total RFO mmoles 100 g^{-1} flour	g 100 g^{-1}flour			
			Sucrose	Raffinose	Stachyose	Verbascose
ILL 1220	Iran	6.1 ± 0.21	1.4 ± 0.13	2.3 ± 0.14	2.9± 0.17	1.3 ± 0.05
ILL 7314	Nepal	5.9 ± 0.23	1.2 ± 0.13	2.3 ± 0.14	2.7± 0.17	1.2 ± 0.05
ILL 5883	Jordan	5.0 ± 0.19	0.9 ± 0.11	2.4 ± 0.14	2.8± 0.17	1.4 ± 0.05
ILL 1553	Iran	5.0 ± 0.23	2.0 ± 0.13	1.7 ± 0.14	2.0± 0.17	1.2 ± 0.05
ILL 4785	Slovakia	4.3 ± 0.19	1.0 ± 0.11	2.4 ± 0.14	2.5± 0.17	1.3 ± 0.05
ILL 4778	Uruguay	4.0 ± 0.23	0.5 ± 0.13	2.7 ± 0.14	2.7± 0.17	1.3 ± 0.05

± indicates standard error of soluble carbohydrate values.

A modified Fiske and SubbaRow (1925) protocol was used to determine the amount of P_i in the supernatant. To each reaction mixture was added 100 µL of 2.5% (w/v) ammonium molybdate dissolved in 2 N HCl and 40 µL of Fiske and SubbaRow reducer. The reaction was incubated for 2 min at room temperature and stopped by addition of 40 µL 34% (w/v) sodium citrate dihydrate solution. The absorbance was measured at 660 nm and the amount of UDP released by GS was determined by using a standard curve (Appendix 6.1a). The standard curve was made with UDP hydrolyzed by apyrase (Ribeiro et al 2000) and correlated to the amount of UDP produced by GS.

6.3.3 Extraction, derivatization and separation of galactinol, myo-inositol and sucrose

Lentil seeds carbohydrates were extracted and separated by gas chromatography using method by Peterbauer et al (1998) with some modifications. Briefly, developing lentil seeds (0.3 g) were ground and extracted with methanol:chloroform:water (12:5:3) at 60 °C for 30 min with pentaerythritol as an internal standard. Phases were separated by addition of one volume of water. An aliquot of the aqueous phase was deionized using ion-exchange resins (Dowex 50-100 mesh; 50 W x 8, H^+ and 1x8, formate) and taken to dryness. Carbohydrates were converted to trimethylsilyl (TMSi) derivatives by treatment with pyridine:N,0-bis-(trimethylsilyl)-trifluoroacetimide:trimethyl-chlorosilane (40:10:1) at 75 °C for 60 min. The TMSi derivatives were separated by GC (HP 5890 Series II, Hewlett-Packard, Vienna, Austria) on a fused silica chemically bonded polydimethylsiloxane column (HP1, 15 m length, 0.53 mm i.d., and 0.15 µm film thickness) with helium as carrier gas at a constant flow of 3 ml min^{-1}. The oven temperature was programmed from 85 °C (1 min isothermal) to 220 °C at 10 °C min^{-1} and further to 325 °C at 12 °C min^{-1}. The final temperature was held at 325 °C for 15 min. The cool-on-column injector was operated in the oven track mode (+3 °C), and the flame ionization detector was held at 330 °C. The retention times of commercial standards of galactinol, myo-inositol and sucrose were used for peak identification and standard curves (Appendix 6.4) for sucrose, galactinol and myo-inositol were used to quantify the individual carbohydrates.

6.4 Results and Discussion

6.4.1 Optimization of GS activity assay

The colorimetric method for the quantification of GS activity assay is based on the indirect determination of the UDP formed from myo-inositol and UDP-gal by the action of GS. To determine UDP formed, it was hydrolyzed by exogenous apyrase releasing Pi which was quantified by a modification of the colorimetric method developed by Fiske and SubbaRow (1925). To find the optimal conditions for the GS assay, reactions were performed using different substrates combination, incubation temperatures and pH. GS specific activity in CDC Redberry seeds (12 DAF) crude extracts determined at different substrate concentrations gave the highest specific activity of 0.1 µmole UDP min^{-1} mg^{-1} protein at 4 mM UDP-gal and 80 mM myo-inositol concentrations (Appendix 6.3a). A seed extract from genotype ILL 4785 (24 DAF) assayed at different temperatures showed increased GS specific activity with increasing temperatures until 45 °C followed by a marked decrease in activity (Appendix 6.3b). A similar peak in GS activity at 50 °C is seen with soybean GS (Ribeiro at al 2000). Determination of pH optimum using genotype ILL 4785 seed extracts (24 DAF) (Appendix 6.3c), gave the highest specific activity (0.032 µmole UDP $min^{-1}mg^{-1}$ of protein) at pH 7.5, which is within GS optimal pH range of 5.5 - 7.5 reported previously (Peterbauer and Richter 2001).

6.4.2 GS activity in lentil genotypes

Specific GS activities in cell crude extracts of lentil seeds with varying RFO concentrations was highest at 16 DAF thereafter decreased as storage proteins were accumulated (Table 6.2). The GS specific activities observed were of the same magnitude as specific activities observed in cell crude extracts of kidney bean (0.0055 µmole $min^{-1}mg^{-1}$) and Zucchini leaf (0.013 µmole $min^{-1}mg^{-1}$) (Liu et al 1995). The highest activity of 113 nmole UDP $min^{-1}mg^{-1}$ of protein was recorded for genotype ILL 1553, followed by 104 nmole UDP $min^{-1}mg^{-1}$ of protein for ILL 5883 (Table 6.2). Both genotypes had medium RFO concentration of 5.1 and 5.0 mmole 100 g^{-1}flour, respectively. GS activity in genotypes ILL 1220 and ILL 7314 with highest RFO concentration were 75 and 22 nmole UDP $min^{-1}mg^{-1}$ of total protein at 16 DAF, respectively. ILL 7314 GS activity of 22 nmole UDP $min^{-1}mg^{-1}$ of total protein was the lowest of all genotypes at 16 DAF. GS activity in seed crude extracts of ILL 4785 and ILL 4778 at 16 DAF was 62 and 84 nmole UDP $min^{-1}mg^{-1}$ of total protein.

At 24 DAF, the GS activity of ILL 1220, ILL 1553, ILL 7314 and ILL 5883 were similar, but GS activity of the low RFO genotypes ILL 4778 and ILL 4785 were considerably

lower than all other genotypes. At 32 DAF, GS activity in lentil seeds of all genotypes was reduced to 2 - 6 nmole UDP $min^{-1}mg^{-1}$ of total protein. At the end of seed development (40 DAF) the high-RFO genotypes showed an increase in specific GS activity whereas the low-RFO genotypes did not indicate any such trend.

6.4.3 Sucrose, galactinol and myo-inositol concentrations and their relationship with total RFO concentration in developing lentil seeds

Sucrose concentration of lentil genotypes ranged from 1.4 - 4.5 g 100 g^{-1} fresh seed weight (FSW) at different stages of seed development (Fig. 6.1a). Sucrose concentration of developing lentil seeds was highest in the early stages of seed development and graduallydecreased as seeds matured. A similar decline in sucrose concentration during seed development has been observed in several other crops (Piotrowicz-Cieslak and Michalczyk 2007). ILL 7314 with the highest total RFO concentration had the highest sucrose concentration at all developmental stages. However, the high-RFO genotype ILL 1220 did not follow a similar trend. The total RFO concentration of ILL 1220 at 16 and 24 DAF were almost equal to sucrose concentration of medium RFO genotypes ILL 1553 and ILL5883. However, at 32 DAF, ILL 1220 had a higher sucrose concentrations than ILL 5883 and ILL1553. Similarly, higher sucrose concentration of 1.6 g 100 g^{-1} in ILL 4785 (lower total RFO concentration) at 40 DAF than 1.4 g 100 g^{-1} sucrose concentration in ILL 1220 (higher total RFO concentration) shows a weak and inconsistent correlation between sucrose and total RFO concentration in developing lentil seeds. Correlation of sucrose with total RFO concentration during seed development stages have not been reported in lentil, however, a moderate correlation (r = 0.49) between sucrose and total RFO concentration in mature seeds have been reported previously (Kernar et al 2004; Tahir et al 2011a)

The galactinol concentration of different lentil genotypes ranged from 0.16 - 0.44 g 100 g^{-1} seed weight at different stages of seed development (Fig 6.1b). The galactinol concentration increased in the middle stages of seed development e.g. 24 and 32 DAF, before showing a decline at 40 DAF. Similar trend of galactinol concentration has been reported in fine-leaved vetch plants (Lahuta et al 2010). At 16 DAF, the highest and lowest galactinol concentrations were observed for accession ILL 1220 and ILL 7314, respectively. These two genotypes had higher total RFO concentration and varied considerably in galactinol concentration at this stage

Table 6.2. Galactnol synthase specific activity in crude cell extract of developing lentil seeds

Genotypes	RFO concentration[a]	GS activity in nmole UDP $min^{-1}mg^{-1}$ protein			
		16 DAF	24 DAF	32 DAF	40 DAF
ILL 7314	High	22 ± 3	15 ± 0	3 ± 1	20 ± 2
ILL 1220	High	75 ± 6	10 ± 2	6 ± 3	9 ± 4
ILL 1553	Medium	113 ± 8	11 ± 0.0	0.2 ± 0.0	10 ± 2
ILL 5883	Medium	104 ± 0.2	14 ± 0.1	2 ± 1	3 ± 1
ILL 4785	Low	62 ± 11	6 ± 0.0	4 ± 1	1 ± 0.0
ILL 4778	Low	84 ± 2	2 ± 0.00	5 ± 4	-

[a] = see Table 6.1,

- = No seeds were obtained

DAF = Days after flowering

of seed development. A variation in GS activity between these two genotypes at 16 DAF was also observed (Table 6.2). The GS activity of 22 nmole UDP $min^{-1}mg^{-1}$ protein in extracts of ILL 7314 was lower than galactinol activity of 75 nmole UDP $min^{-1}mg^{-1}$ protein observed for ILL 1220 at 16 DAF. The variation observed in galactinol concentration may, therefore, be due to differences in GS activity. At 24 DAF, a small difference in galactinol concentrations of ILL 7314 and ILL 1220, i.e. 0.28 and 0.29 g 100 g^{-1} seed weight may be due to higher GS activity of 15 nmole UDP $min^{-1}mg^{-1}$ protein observed for ILL 7314 compared to 10 nmole UDP $min^{-1}mg^{-1}$ protein for ILL 1220. Similarly, at 40 DAF the higher galactinol concentration of ILL 7314 may be attributed to higher GS activity of 20 nmole UDP $min^{-1}mg^{-1}$ protein compared to 9 nmole UDP $min^{-1}mg^{-1}$ protein observed for ILL 1220.

Differences in galactinol concentration in genotypes with medium and low RFO concentration were small at 16 DAF and ranged from 0.25 - 0.28 g 100 g^{-1}seed weight (Fig 6.1). Higher GS activity of 113 and 104 nmole UDP $min^{-1}mg^{-1}$ protein in medium RFO genotypes, e.g. ILL 1553 and ILL 5883, respectively, compared to lower total RFO group genotypes did not produce higher galactinol concentration as was expected. However, potential use of galactinol in other biosynthetic pathways especially in biosynthesis of galactosyl cyclitols as galactosyl donor complicates the correlation between GS activity and galactinol concentration. At 24 DAF a significant increase in galactinol concentration was observed in all genotypes with concentrations ranging from 0.28 - 0.44 g 100 g^{-1}. A higher galactinol concentration of 0.44 and 0.31 g 100 g^{-1} was observed in ILL 5883 and ILL 1553, respectively, compared to other lentil genotypes. Galactinol concentration in ILL 1220, ILL 7314 and ILL 4778 were similar at 24 DAF. The higher galactinol concentration in ILL 5883 of 0.44 g 100 g^{-1} is difficult to explain since GS activity in ILL 5883 extract was not very different from other genotypes. However, the higher GS activity observed for ILL5883 at 16 DAF may provide a reason for the higher galactinol concentration. At 32 DAF the galactinol concentration ranged from 0.27 - 0.42 g 100 g^{-1} among different genotypes. Overall, an increase in galactinol concentration was observed for four genotypes i.e. ILL 7314, ILL 1220, ILL 1553 and ILL 4778, whereas a decrease was recorded for two genotypes i.e. ILL 5883 and ILL 4785, compared to galactinol concentrations at 24 DAF. At 40 DAF galactinol concentration of various genotypes ranged from 0.21 - 0.37 g 100 g^{-1} and reduced slightly compared to galactinol concentration at 24 and 32 DAF. The variation in

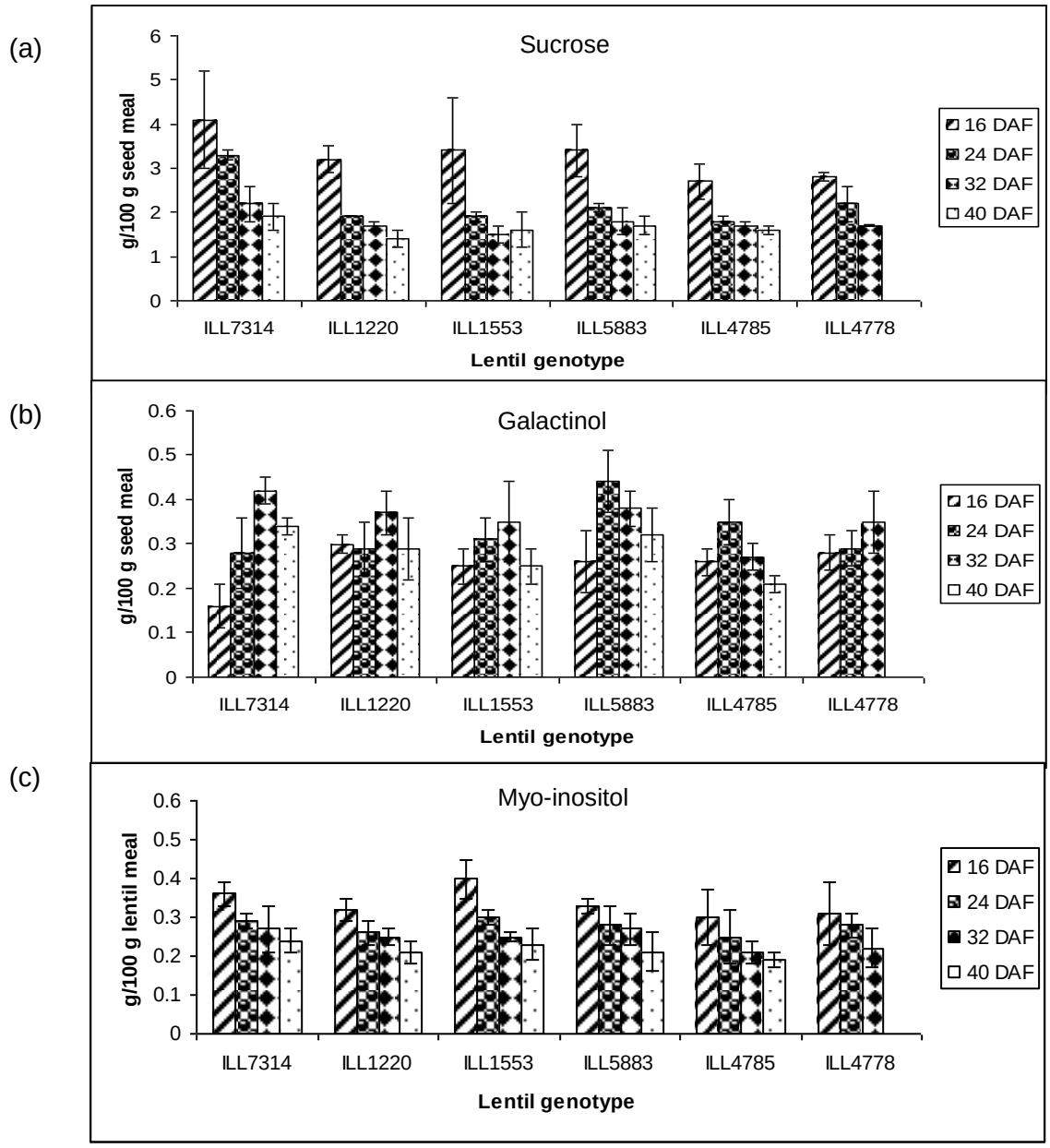

Figure 6.1. Sucrose (a) galactinol (b) and myo-inositol (c) concentrations in developing seeds of lentil at different days after flowering (DAF).

the galactinol concentrations in different genotypes at different stages of seed development e.g. 16, 24, 32 and 40 DAF does not show a clear association between the amount of galactinol produced and the total RFO concentration of seeds. Similarly, differences in GS activities at different stages do not show a clear relationship between GS and RFO concentration in lentil seeds. The reduction in galactinol concentration in the later phases of seed development may be attributed to their use in total RFO synthesis which commences at the later stages of seeds development when seeds start to desiccate. The findings from this study suggest that GS and galactinol concentration may not play a regulatory role in RFO biosynthesis. It also suggests that the role of other enzymes particularly enzymes involved in galactosyl cyclitols and/or a shared RFO and galactosyl cyclitols pathway and the presence of other galactosyl donors in the RFO biosynthetic pathway.

The myo-inositol concentration in different lentil genotypes ranged from 0.19 - 0.40 g 100 g^{-1} meal at different selected stages of seed development (Fig. 6.1c). The myo-inositol concentrations were higher in the initial phases and gradually reduced with seed development. The myo-inositol concentration at 16 DAF ranged from 0.30 - 0.40 g 100 g^{-1}. The highest and the lowest myo-inositol concentration were observed in ILL 1553 and ILL 4785 at 16 DAF. ILL 1553 belonged to the medium total RFO group. The myo-inositol concentration of ILL 4785 and ILL 4778, both in the low total RFO group, were lower than all other genotypes at 16 DAF. At 24, 32 and 40 DAF, the myo-inositol concentration in developing lentil seeds ranged from 0.25 - 0.30 g, 0.21 - 0.27 g, and 0.19 - 0.24 g 100 g^{-1}, respectively. Although the differences in myo-inositol concentration of higher and medium total RFO groups were small, but consistent lower myo-inositol concentration of lower RFO group genotype i.e.ILL 4785 and ILL 4778 indicates a positive association between myo-inositol and total RFO concentration in lentil seeds. A similar, positive correlation between myo-inositol and total RFO concentration has been reported previously (Karnar et al 2004). However, Lahuta et al (2010) reported no such association between myo-inositol and total RFO biosynthesis. The feeding of elevated levels of sucrose and myo-inositols to fine leaved vetch explants unexpectedly promoted accumulation of galactosyl pinitols but not of RFO (Lahuta et al 2010), thus contradicting such association as well as regulatory role of myo-inositol in RFO biosynthesis.

Association among soluble sugars varied during different phases of lentil seed development. Principle component analysis (PCA) identifies patterns and provides a visual

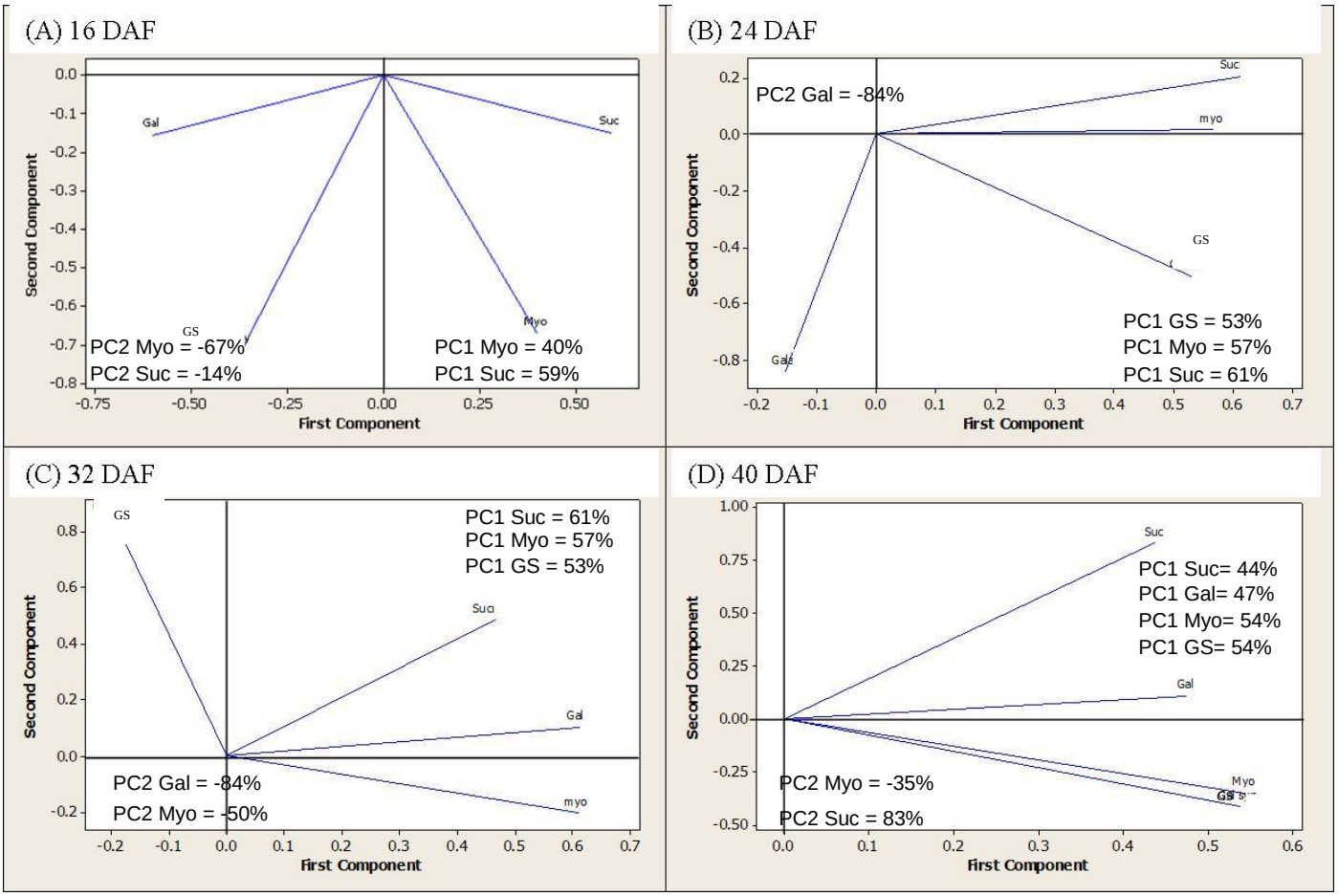

Figure 6.2. Plots of major principal components showing relationship between soluble carbohydrates in developing lentil seeds at different days after flowering (DAF).
Abbrevations: (Gal: galactinol, GS: galactinol synthase activity, Suc: sucrose, Myo: myo-inositol.

Table 6.3. Summary of correlations between soluble carbohydrates at different stages of lentil seed development.

	16 DAF	24 DAF	32 DAF	40DAF
Sucrose-Myo-inositol	+	+	+	+
Myo-inositol-Galactinol	-	-	+	+
Galactinol-Sucrose	-	-	+	+
GS activity - Galactinol	+	+	-	+

+ indicates a positive association; – indicates an inverse relationship.

representation of the data (Fig 6.2). In a PCA plot, an association between two or more variable is shown when the variables cluser in the same quadrant. A lack of association between variables is indiacted when the variables are scattered in all direction. At 16 DAF a significant ($P \leq 0.05$) negative association ($r = -0.79$) was found between galactinol and sucrose concentration. Association between sucrose and myo-inositol concentration and galactinol synthase activity and galactinol concentration, though positively, were not significant ($P \leq 0.05$) (Fig. 6.2 A). At 24 DAF, a positive but weak association existed between sucrose and myo-inositol concentration and galactinol synthase activity and galactinol concentration (Fig 6.2 B). At 32 DAF, a significant ($P \leq 0.05$) correlation ($r = 0.87$) between galactinol and myo-inositol concentration was observed. Similar association between galactinol and myo-inositol concentration were reported in pea seeds (Karner et al 2004). Galactinol synthase activity and galactinol concentration, however, were indendent of each other and this lack of association may be due to the significant reduction in galactinol synthase activity observed at this stage of seed developement (Fig. 6.2 C). Associations between other soluble carbohydrates were not significant at this stage of seed development. A significant ($P \leq 0.05$) positive association ($r = 0.9$) between galactinol synthase activity and myo-inositol concentration was observed at 40 DAF. This positive correlation is indiacted by the close position of the lines of galactinol synthase and myo-inositol in the same quadrant in the PCA plot at 40 DAF (Fig.6.2 D). Correlations between other soluble carbohydrates though positive were not significant ($P \leq 0.05$). The correlation among galactinol synthase activity and other soluble carbohydrates during different stages of seed development are summarized in Table 6.3.

6.4.4 Summary and Conclusions

In conclusion an association between GS specific activity, sucrose and galactinol concentrations at different stages of seed development with the total RFO concentration of mature seeds was not observed. However, a positive association was observed between myo-inositol concentration and total RFO concentration may affect total RFO concentration in lentil seeds. Results from this study suggest a non regulatory role for GS and galactinol concentration in RFO biosynthesis. Furthermore, it may also indicate the role of other galactosyl donor beside galactinol in RFO biosynthetic pathway.

7 GENERAL DISCUSSION

7.1 Research findings

The seed composition analysis showed significant variation in total RFO concentration and composition in selected lentil genotypes (Chapter 3). The Pearson correlation coefficient (r) was used as a measure of degree of relationship between different quality traits. The absence of any significant correlation between total RFO and other important quality traits such as starch and protein concentration suggest that selection for low RFO concentration may not affect these important seed quality parameters in lentil. Therefore, the variation in RFO concentration may be utilized to develop lentil cultivars with reduced RFO concentration. The results of trait correlation analysis and the values of correlation coefficient (r) are interpreted in terms of negligible (r = 0.0 - 0.2), weak (r = 0.2 - 0.4), moderate (r = 0.4 - 0.7), strong (r = 0.7 - 0.9), and perfect (r = 1.0) relationship. Based on this interpretation, the positive correlation between RFO concentration and seed weight as well as between starch concentration and seed weight were classified as moderate. Similarly, negative correlation between starch and protein concentration as well as between seed weight and amylose concentration were classified as moderate associations. Relationships among the rest of measured seed constituents were negligible or weak. Decortication of lentil seeds significantly increased the starch concentration. Similar, increase in starch concentration as well as an increase in protein, stachyose and verbascose concentration and a decrease in tannin, sucrose and raffinose concentrations as a result of decortication of lentils have been have been previously reported (Wang 2008). Decortication, however, also results in losses in the form of broken seeds and powder, and therefore an efficient and improved method for decortication is an important objective for lentil processors and breeders.

The different environmental conditions used in the study had significant effects on soluble carbohydrates in lentil cultivars, but the variayion in total RFO concentration was not large from a lentil improvement perspective (Chapter 4). The negative association observed between precipitation received during lentil growing season and total RFO concentration of lentil cultivars may indicate that effect plant water status affects total RFO accumulation and the physiological role of RFO in plants. The high broad sense heritability of total RFO and sucrose

estimated from the components of variance indicated that total RFO trait is highly heritable and that lentil total RFO concentration may be reduced by selection and crossing of low RFO lentil genotypes through conventional breeding. The significant positive correlation between total RFO and sucrose found in our study confirm a similar finding by Karner et al (2004). Similarly, results from this study also agree with a recent study showing variation in RFO content of soybean cultivars grown at different geographical locations (Kumar et al 2010) and several other studies where different environmental factors were shown to affect soluble carbohydrates contents in plants (Gilmour et al 2000, Taji et al 2002).

An evaluation of natural genetic variation and diversity in total RFO concentration and composition of individual oligosaccharides in genus *Lens* is important for identification of low-RFO germplasm for lentil improvement (Chapter 5). Our assessment of the lentil germplasm collection showed more variation and genetic diversity in total RFO concentration and composition in wild lentils as compared to domesticated genotypes. *Lens ervoides* genotype ILWL 285 and *Lens culinaris ssp. culinaris* genotype ILL 4778 with the lowest total RFO concentration in wild and domesticated genotypes, respectively, and several other genotypes with varying RFO concentrations identified as a result of this study may provide the variation needed for development of lentil cultivars with low total RFO concentration. Similarly, *Lens ervoides* genotypes with higher verbascose and lower stachyose concentration and *Lens nigricans* genotypes with higher raffinose and lower verbascose concentration identified in this study may be used for understanding the variation in RFO biosynthetic pathway.

The mechanisms regulating the biosynthesis and accumulation of RFO and other galactosyl cyclitols in plant seeds are not well understood. The role of GS has drawn the interest of researchers for its possible regulatory role in RFO biosynthetic pathway. Correlation between GS activity and accumulation of RFO in plants (Handley et al 1983; Saravitz et al 1987) has been contradictory and has not been studied in lentil seeds. Therefore the fourth major objective of our study was to determine if GS activity correlates with RFO concentration in lentil seeds. The results of our study (Chapter 6) did not indicate any association between GS activity and RFO concentration during lentil seeds developement. GS activity at different stages of seed development in lentil seeds with higher total RFO concentration was less than galactinol activity observed in seeds with lower total RFO concentration. These results concur with a study of pea seeds where GS activity and RFO composition were unrelated (Peterbauer et al 2001).

Furthermore, galactinol and sucrose concentrations of different lentil genotypes at different developing stages were also not associated with total RFO concentration of the seeds.

7.2 Comparison of RFO quantification and composition methods and selection of lentil genotypes

The analysis of large numbers of samples required robust yet effective methods for measuring RFO concentration and composition in lentil seed extracts. The enzymatic method of total RFO quantification was used to provide repeatable quantitative measurements. The major limitation of this method, however, is the quantification of total RFO concentration as a group in mmoles 100 g^{-1} rather than as individual oligosaccharides in a sample. The availability of equipments for the enzymatic method compared to gas chromatography and sensitive HPLC based methods and overall suitability of this method for our objectives was a major factor in the selection of the enzymatic method for RFO analysis. The HPLC-SEC method for RFO composition analysis using a Sugar-Pak column coupled with a refractive index detector provided a good resolution of chromatographic peaks. For evaluation of these methods RFO concentrations of domesticated accessions obtained using HPLC-SEC converted in mmoles 100 g^{-1}, summed up and compared with RFO concentration obtained from the enzymatic method (Table 7.1). Our results of the comparison of the two methods indicated higher total RFO values for the enzymatic method than the HPLC-SEC based method. Out of 122 domesticated accessions 76 and 45 accessions had total concentration differences of 00 - 1.00 mmoles and 1.1 - 1.9 mmoles 100 g^{-1}, respectively. These differences may due to the hydrolysis of galactosyl derivatives of cyclitols e.g. ciceritol present in lentil seeds. Galactosyl cyclitols contain α-(1→6) linkages similar to RFO that are hydrolyzed by α-galactosidases and may be the cause of higher concentrations of RFO estimated by the enzymatic method (Fig. 2.4, Table 7.1). Similar results were reported by Kumar et al (2010) in soybean seeds where higher concentrations of total RFO were obtained by the enzymatic method compared to HPLC based method.

HP-SEC was used for composition analysis of soluble carbohydrates in lentil seeds extracts. Sugar standards of verbascose, stachyose, raffinose, sucrose and glucose were available and were used for identification of chromatographic peaks. Some chromatographic peaks, however, were not identified and to find the percentage area of the unresolved peaks the peak area of identified soluble carbohydrates were summed and compared (Table 7.2). The peak area

Table 7.1. Total RFO concentration in domesticated lentil genotypes.

Country of origin	Province	Genotype	Total RFO	
			Megazyme Method	HP-SEC Method
Afghanistan	Paravan	ILL 1762	5.1	4.1
Afghanistan	Farah	ILL 213	5.2	4.3
Afghanistan	Heart	ILL 7567	5.2	4.0
Afghanistan		ILL 2217	5.4	3.9
Argentina		ILL 4605	4.9	4.2
Armenia	Artik	ILL 86	4.9	4.1
Azerbaijan	Nachichevan	ILL 1671	5.4	3.9
Bangladesh	Gopalganj district	ILL 7773	5.3	3.9
Bulgaria		ILL 5399	5.0	4.4
Brazil		ILL 6967	5.1	3.7
Canada		ILL 4349	5.2	4.1
Chile		ILL 2290	5.2	4.3
Chile		ILL 5490	5.3	4.4
China		ILL 7499	5.6	4.2
Colombia		ILL 1649	5.7	4.2
Cyprus	Limassol	ILL 5968	5.1	4.1
Czech republic		ILL 4783	5.4	4.1
Germany		ILL 4831	4.5	4.0
Algeria	Batna	ILL 7051	5.2	3.9
Egypt	Marsa Matruh	ILL 6540	4.3	3.9
Egypt		ILL 784	5.3	4.5
Egypt	El Giza	ILL 4387	5.5	4.0
Spain	Castilla la Mancha	ILL 80	5.1	4.6
Spain	Castilla la Mancha	ILL 5058	5.4	3.9
Ethiopia		ILL 1983	4.9	3.8
Ethiopia	Gonder	ILL 5945	5.0	4.1
Ethiopia	Sidama	ILL 2433	5.1	4.3
Ethiopia	Shewa	ILL 1683	5.4	4.3
Ethiopia	Shewa	ILL 1744	5.6	4.4
France	Center	ILL 4740	5.2	4.4
Greece	Peloponnesus	ILL 4865	4.5	4.2
Greece	Ionian Islands	ILL 293	4.9	4.5
Guatemala		ILL 494	4.9	4.2
Croatia	Croatia	ILL 4915	5.3	3.9
Hungary		ILL 4665	5.3	4.1
India		ILL 4359	4.1	3.9
India		ILL 3596	4.4	3.2
India		ILL 2684	4.5	3.7
India		ILL 3597	4.5	3.2
India		ILL 2501	4.6	4.0
India		ILL 2789	4.6	3.7
India		ILL 3805	4.6	3.3

Country of origin	Province	Genotype	Total RFO	
			Megazyme Method	HP-SEC Method
India		ILL 2581	4.7	3.9
India		ILL 3347	4.7	3.2
India		ILL 4080	4.7	3.7
India		ILL 2526	4.8	3.6
India		ILL 3925	4.8	4.1
India		ILL 2607	4.9	3.2
India		ILL 3025	4.9	3.8
India		ILL 3714	4.9	3.1
India	Uttar Pradesh	ILL 132	5.1	4.4
India		ILL 4164	5.1	4.4
India	West Bengal	ILL 5151	5.1	4.1
India		ILL 3167	5.2	3.3
India	West Bengal	ILL 5080	5.6	4.4
Iran		ILL 2372	4.9	3.9
Iran	Esfahan	ILL 1553	5.0	3.1
Iran	Fars	ILL 242	5.2	4.2
Iran	Mazandaran	ILL 871	5.4	4.6
Iran	Kerman	ILL 1462	5.4	3.8
Iran	Gilan	ILL 4903	5.4	3.9
Iran	East Azerbaijan	ILL 7621	5.4	3.9
Iran	Khorasan	ILL 1337	5.6	4.3
Iran	Fars	ILL 1048	5.8	3.9
Iran	Fars	ILL 1220	6.1	4.2
Iraq	Dahuk	ILL 55	4.7	4.4
Italy	Puglia	ILL 5418	4.8	4.1
Jordan		ILL 5883	5.0	4.2
Jordan	Ma'an	ILL 6920	5.0	4.2
Jordan	Karak	ILL 9	5.2	4.6
Jordan	Karak	ILL 5584	5.2	4.3
Jordan	Irbid	ILL 5209	5.3	4.4
Lebanon		ILL 1139	5.3	3.4
Libya		ILL 4804	4.9	4.2
Morocco	Centre Nord	ILL 6505	4.8	3.9
Morocco	Centre	ILL 7727	4.8	3.9
Mexico		ILL 5425	4.7	4.2
Macedonia	Macedonia	ILL 624	4.9	4.4
Netherlands		ILL 4609	4.7	3.8
Norway		ILL 4782	5.2	4.2
Nepal	Narayani	ILL 7791	4.4	3.6
Nepal	Janakpur	ILL 3487	4.5	2.7
Nepal	Janakpur	ILL 7314	5.9	4.0
Pakistan	NWF	ILL 6378	4.8	3.9
Pakistan	NWF	ILL 229	5.1	4.3

Country of origin	Province	Genotype	Total RFO	
			Megazyme Method	HP-SEC Method
Pakistan	Northern Areas	ILL 2194	5.7	4.1
Palestine	Haifa	ILL 313	5.0	5.0
Poland	Lublin	ILL 5424	4.5	4.4
Portugal		ILL 4956	4.6	3.9
Romania		ILL 4774	4.8	4.1
Russia	Tambov oblast	ILL 7089	4.2	3.7
Russia		ILL 2191	4.7	4.3
Saudia Arabia	Al-Baha, Aseer	ILL 7745	4.7	3.6
Serbia	Sebia	ILL 5576	4.6	4.6
Sudan	Al Khartum	ILL 1861	5.2	4.4
Slovakia		ILL 4785	4.3	4.0
Syria	Aleppo	ILL 7747	4.8	3.4
Syria	Al Hasakah	ILL 5511	4.9	4.1
Syria	Al Qunaytirah	ILL 6689	4.9	4.4
Syria	Lattakia	ILL 6853	4.9	4.0
Syria	Homs	ILL 28	5.0	4.6
Syria	Al Hasakah	ILL 4542	5.0	4.2
Syria		ILL 4400	5.3	4.4
Tajikistan		ILL 618	4.9	3.9
Tunisia		ILL 6182	4.4	3.9
Turkey	Nigde	ILL 7585	4.5	3.9
Turkey	Tunceli	ILL 141	4.7	4.3
Turkey	Hakkari	ILL 2171	4.9	3.9
Turkey	Kastamonu	ILL 927	5.1	3.9
Turkey	Corum	ILL 6166	5.2	3.8
Turkey	Tunceli	ILL 572	5.8	3.9
United Kingdom	Kiev	ILL 595	5.1	4.3
Unknown		ILL 3502	5.0	3.0
Uruguay		ILL 4778	4.0	4.2
USA		ILL 4671	5.1	4.1
Uzbekistan		ILL 4875	5.2	4.4
Yemen	San'a	ILL 4768	4.7	4.0
Yugoslavia		ILL 2230	5.1	4.0
		ILL 5845	5.3	4.4
		ILL 6264	5.1	4.1
		ILL 9901	4.9	4.9
		ILL 10021	4.9	3.6
Std Error			0.19 - 0.26	
Range			4.0 - 6.1	

Table 7.2. Analysis of soluble carbohydrates in cell extracts of lentil seeds.

Country of origin	Genotype	Peak area* (%)							
		Verbascose	Stachyose	Raffinose	TRFO	Sucrose	Glucose	Total identified	Unidentified
Argentina	ILL 9	4.2	22.0	16.3	42.5	23.9	1.2	67.6	32.5
Poland	ILL 28	4.4	20.5	15.5	40.3	23.8	1.9	66.0	34.1
France	ILL 55	4.7	21.8	16.9	43.3	22.1	1.4	66.8	33.3
Egypt	ILL 80	4.5	20.2	15.2	39.9	24.6	1.7	66.1	34.0
Egypt	ILL 86	2.8	18.2	15.0	35.9	30.1	2.1	68.0	32.0
India	ILL 132	3.3	21.1	17.3	41.7	24.4	1.6	67.6	32.5
Croatia	ILL 141	2.9	22.1	17.1	42.0	24.2	1.4	67.6	32.4
India	ILL 213	4.1	21.3	14.1	39.4	26.9	2.3	68.7	31.4
India	ILL 229	2.8	23.1	16.5	42.4	23.6	1.9	67.8	32.2
Guatemala	ILL 242	3.1	20.9	15.3	39.3	25.7	1.4	66.3	33.8
Ethiopia	ILL 293	3.0	21.9	16.2	41.1	23.6	2.0	66.7	33.3
Germany	ILL 313	3.9	22.4	16.4	42.8	22.4	1.3	66.5	33.5
Russia	ILL 494	3.5	21.4	16.3	41.1	22.2	2.0	65.3	34.7
Iran	ILL 572	1.5	22.0	14.8	38.3	25.4	1.6	65.2	34.9
Nepal	ILL 595	4.1	22.2	15.6	41.8	22.0	1.8	65.7	34.4
Saudia Arabia	ILL 618	3.7	18.8	14.5	37.0	27.3	1.2	65.5	34.6
Syria	ILL 624	4.1	22.4	17.2	43.7	20.0	1.9	65.6	34.5
Turkey	ILL 784	5.2	24.1	16.6	45.9	19.2	1.6	66.7	33.3
Jordan	ILL 871	3.9	19.8	14.2	37.9	27.5	2.1	67.4	32.7
Syria	ILL 927	2.1	18.8	11.5	32.4	32.6	1.6	66.5	33.5
Mexico	ILL 1048	1.9	22.7	15.5	40.0	25.3	1.4	66.7	33.3
Turkey	ILL 1139	3.2	22.4	12.7	38.4	28.2	1.4	67.9	32.1
India	ILL 1220	3.4	23.0	14.8	41.1	24.2	1.8	67.1	33.0
Portugal	ILL 1337	4.6	22.1	14.7	41.3	26.7	1.4	69.4	30.6
Jordan	ILL 1462	3.9	22.9	14.8	41.6	24.8	1.7	68.1	32.0

Country of origin	Genotype	Peak area* (%)							
		Verbascose	Stachyose	Raffinose	TRFO	Sucrose	Glucose	Total identified	Unidentified
Afghanistan	ILL 1553	3.1	20.6	14.0	37.7	30.5	1.7	70.0	30.0
Yemen	ILL 1649	2.3	22.5	15.6	40.4	24.5	1.9	66.8	33.2
Iran	ILL 1671	3.2	20.5	13.9	37.5	27.6	1.6	66.6	33.5
Iran	ILL 1683	3.9	21.5	17.4	42.8	23.3	1.6	67.8	32.3
Spain	ILL 1744	5.4	24.4	15.9	45.7	20.6	1.7	68.0	32.0
Armenia	ILL 1762	3.4	22.3	16.6	42.3	25.6	2.3	70.1	29.9
India	ILL 1861	4.5	24.1	16.8	45.4	21.7	1.7	68.7	31.3
Iran	ILL 1983	3.5	23.1	16.7	43.3	23.9	2.7	69.8	30.2
Jordan	ILL 2171	4.7	22.1	17.4	44.1	21.9	2.2	68.2	31.8
Italy	ILL 2191	4.1	19.6	18.2	41.9	24.4	2.4	68.7	31.3
Slovakia	ILL 2194	2.2	21.9	17.1	41.3	27.1	2.0	70.4	29.7
Syria	ILL 2217	1.8	20.2	16.7	38.7	27.2	2.7	68.6	31.5
Ethiopia	ILL 2230	3.2	21.8	18.0	42.9	23.1	2.5	68.4	31.6
Iraq	ILL 2290	3.3	20.2	17.5	41.1	24.8	2.2	68.0	32.0
Macedonia	ILL 2372	3.1	18.9	15.9	37.9	28.8	2.2	68.9	31.2
Ethiopia	ILL 2433	4.8	23.7	18.8	47.1	21.9	1.8	70.7	29.3
India	ILL 2501	3.2	21.6	18.6	43.3	22.8	3.7	69.8	30.2
Nepal	ILL 2526	3.1	21.1	17.9	42.1	26.2	2.9	71.2	28.9
India	ILL 2581	3.2	20.7	18.1	42.1	25.5	3.1	70.7	29.4
Netherlands	ILL 2607	3.9	20.7	16.0	40.6	24.4	2.8	67.9	32.1
USA	ILL 2684	3.4	19.7	17.1	40.2	25.0	3.1	68.3	31.7
Ethiopia	ILL 2789	3.7	20.6	17.4	41.7	25.0	3.0	69.7	30.3
Iran	ILL 3025	3.2	20.6	18.6	42.3	24.4	3.0	69.7	30.3
Russia	ILL 3167	4.3	24.2	19.3	47.9	.	.	.	.
India	ILL 3347	3.1	20.1	17.1	40.4	24.7	3.5	68.6	31.4
Yugoslavia	ILL 3487	2.5	21.7	18.4	42.6	.	.	.	.
Afghanistan	ILL 3502	2.1	19.2	16.5	37.9	24.1	2.16	64.2	35.8

Country of origin	Genotype	Peak area* (%)							
		Verbascose	Stachyose	Raffinose	TRFO	Sucrose	Glucose	Total identified	Unidentified
Hungary	ILL 3596	2.9	20.4	17.0	40.3	25.7	2.7	68.7	31.3
China	ILL 3597	2.9	20.8	18.9	42.5	.	.	.	.
Turkey	ILL 3714	2.4	21.1	17.1	40.6	25.7	3.0	69.3	30.7
Romania	ILL 3805	3.6	21.6	18.1	43.3	.	.	.	.
United Kingdom	ILL 3925	3.2	21.0	17.6	41.8	23.3	2.6	67.6	32.4
India	ILL 4080	3.1	19.8	18.1	40.9	25.6	2.7	69.2	30.8
Syria	ILL 4164	4.8	23.2	19.3	47.3	23.0	1.8	72.1	28.0
Syria	ILL 4349	4.7	19.9	16.1	40.7	24.8	2.6	68.0	32.0
Afghnistan	ILL 4359	3.8	20.3	17.3	41.3	23.5	3.0	67.8	32.2
Czech Republic	ILL 4387	3.1	20.8	16.2	40.1	26.1	2.2	68.4	31.7
Turkey	ILL 4400	4.9	19.3	17.1	41.2	26.2	2.0	69.4	30.7
India	ILL 4542	5.1	20.5	18.1	43.6	22.6	1.9	68.0	32.0
Uruguay	ILL 4605	5.1	20.2	15.5	40.7	26.7	1.9	69.3	30.7
Morocco	ILL 4609	4.6	19.7	17.3	41.5	24.3	2.7	68.5	31.5
Chile	ILL 4665	3.9	21.0	17.2	42.1	23.8	1.7	67.6	32.5
India	ILL 4671	4.7	20.6	15.3	40.6	25.1	2.0	67.6	32.4
Iran	ILL 4740	2.6	24.4	18.6	45.6	19.2	2.0	66.7	33.3
Azerbaijan	ILL 4768	4.2	20.8	18.1	43.0	24.3	1.9	69.2	30.8
Romania	ILL 4774	3.1	21.1	17.3	41.5	23.1	1.9	66.5	33.6
Sudan	ILL 4778	4.4	20.9	20.6	45.8	18.7	2.8	67.3	32.8
Tunisia	ILL 4782	3.6	20.7	17.7	42.0	25.7	2.2	69.9	30.2
Iran	ILL 4783	3.5	22.8	17.6	43.9	23.0	1.9	68.8	31.3
Nepal	ILL 4785	4.3	21.1	19.0	44.3	23.0	2.0	69.3	30.8
Jordan	ILL 4804	3.8	22.3	16.5	42.6	22.3	1.7	66.5	33.6
Canada	ILL 4831	3.7	22.2	17.7	43.5	24.0	2.6	70.1	29.9
India	ILL 4865	3.7	22.4	18.1	44.1	23.4	1.7	69.2	30.8

Country of origin	Genotype	Peak area* (%)							
		Verbascose	Stachyose	Raffinose	TRFO	Sucrose	Glucose	Total identified	Unidentified
Pakistan	ILL 4875	4.1	21.9	16.0	41.9	26.2	3.0	71.0	29.0
Pakistan	ILL 4903	4.2	20.3	15.0	39.6	28.1	1.8	69.4	30.7
Iran	ILL 4915	4.1	21.0	17.3	42.3	22.4	2.5	67.1	32.9
Colombia	ILL 4956	4.5	21.6	17.5	43.5	21.4	2.0	66.8	33.2
India	ILL 5058	2.6	20.0	16.7	39.2	26.5	2.1	67.8	32.2
India	ILL 5080	5.5	24.2	19.3	49.0	18.7	2.1	69.7	30.3
Greece	ILL 5151	4.5	23.4	18.1	46.0	23.2	2.0	71.2	28.8
Greece	ILL 5209	5.1	23.6	19.3	48.0	23.5	1.6	73.1	26.9
Pakistan	ILL 5399	4.6	23.2	18.2	46.1	19.8	2.0	67.9	32.1
India	ILL 5418	4.4	20.6	18.3	43.4	21.5	2.5	67.3	32.7
Turkey	ILL 5424	2.7	20.0	21.6	44.3	20.6	2.0	66.8	33.2
Afghanistan	ILL 5425	2.9	20.5	19.2	42.7	21.0	1.4	65.0	35.0
India	ILL 5490	4.4	21.2	16.6	42.1	24.4	2.0	68.5	31.6
Lebanon	ILL 5511	4.5	20.0	17.7	42.1	24.5	2.0	68.5	31.5
Unknown	ILL 5576	3.3	18.1	15.5	36.9	26.8	1.8	65.4	34.6
Uzbekistan	ILL 5584	5.3	22.1	18.2	45.5	23.9	1.6	71.0	29.0
Brazil	ILL 5845	4.7	20.6	18.6	43.8	23.3	2.0	69.2	30.9
Tajikistan	ILL 5883	4.6	22.1	17.3	44.0	22.9	2.0	68.8	31.2
Serbia	ILL 5945	4.4	21.6	18.6	44.5	25.0	2.0	71.4	28.6
Cyprus	ILL 5968	3.1	22.2	17.2	42.5	26.6	1.9	70.9	29.1
Syria	ILL 6166	2.2	20.1	16.7	39.0	27.0	2.0	67.9	32.1
	ILL 6182	3.9	20.2	17.6	41.8	23.6	2.0	67.3	32.8
Algeria	ILL 6264	4.8	22.0	17.4	44.2	23.7	2.1	69.9	30.2
Bulgaria	ILL 6378	3.4	18.1	17.1	38.6	28.6	2.8	69.9	30.1
India	ILL 6505	2.9	18.2	17.1	38.1	25.1	3.3	66.5	33.5
Egypt	ILL 6540	3.0	20.7	15.6	39.3	26.9	2.1	68.2	31.8
India	ILL 6689	6.2	22.5	17.1	45.8	20.1	1.9	67.8	32.2

Country of origin	Genotype	Peak area* (%)							
		Verbascose	Stachyose	Raffinose	TRFO	Sucrose	Glucose	Total identified	Unidentified
Palestine	ILL 6853	3.7	18.8	18.3	40.7	28.8	2.7	72.2	27.9
Norway	ILL 6920	4.1	20.3	19.0	43.4	27.7	1.9	73.0	27.1
Chile	ILL 6967	3.3	18.5	17.5	39.2	30.2	2.8	72.1	27.9
Spain	ILL 7051	4.9	19.0	15.7	39.5	29.0	2.2	70.6	29.4
Iran	ILL 7089	3.9	18.2	18.0	40.1	24.1	3.4	67.6	32.4
Morocco	ILL 7314	2.4	21.7	16.9	41.0	26.5	2.4	69.9	30.1
	ILL 7499	2.3	23.1	18.6	44.0	24.7	2.0	70.6	29.4
Iran	ILL 7567	4.8	19.5	16.7	41.0	26.4	2.1	69.5	30.6
Libya	ILL 7621	4.2	19.0	15.9	39.1	27.8	2.6	69.4	30.6
Turkey	ILL 7585	4.0	19.8	17.5	41.2	25.2	3.2	69.6	30.5
Syria	ILL 7727	5.1	20.5	18.5	44.1	24.5	2.2	70.8	29.2
India	ILL 7745	4.6	20.4	18.1	43.0	24.8	2.4	70.2	29.8
Bangladesh	ILL 7747	3.1	20.9	19.1	43.1	26.1	2.4	71.6	28.5
India	ILL 7773	6.0	22.5	18.9	47.4	20.3	2.2	69.8	30.2
India	ILL 7791	2.6	20.4	19.7	42.7	26.0	2.0	70.7	29.4
Ethiopia	ILL 9901	3.9	20.5	16.7	41.1	.	.	.	.
Jordan	ILL 10021	5.3	21.9	16.7	44.0	.	.	.	.
	Range	1.5 - 6.2	18.1 - 24.4	11.5 - 21.6	32.4 - 49.0	18.7 - 30.2	1.6 - 3.7	64.2 - 73.1	26.9 - 35.8

* Soluble carbohydrates were determined by HPLC-SEC and the area under each carbohydrate was integrated by Empower software as described on page 52.

of verbascose, stachyose and raffinose ranged from 1.5 - 6.2%, 18.1 - 24.4% and 11.5 - 21.6%, respectively. The total RFO obtained by adding the values of verbascose, stachyose and raffinose ranged from 32.4 - 49.0% in lentil seed extracts. Similarly, sucrose and glucose peaks areas in lentil seed extract ranged from 18.7 - 30.2% and 1.6 - 3.7%, respectively. Fructose, however, was not identified in most lentil seed extracts due to its low concentration. The total soluble carbohydrate concentration, calculated by adding the peak area of all resolved peaks, ranged from 64.2 - 73.1% of the chromatographic area whereas the unknown peak area ranged from 26.9 - 35.8% in seed extracts in different lentil accessions.

Selection of plant material for experimentation is an important step in any research work. For our first study (Chapter 3) lentil genotypes were selected to give a broad overview of the variation in quality traits and especially RFO concentration and composition in lentil cultivars, breeding lines and accessions that have been used or are currently used in lentil improvement programs in the Crop Development Center (CDC) at the University of Saskatchewan. Similarly, lentil cultivars developed, grown and adapted to growing condition in Saskatchewan were used to assess the effect of environmental condition on RFO concentration (Chapter 4). For evaluation of variation in RFO concentration in genus *Lens* (Chapter 5), ICARDA lentil germplasm collection was used for representing genus Lens from all geographical regions of the world.

7.3 Future research

Different species and subspecies of lentil and germplasm from different regions were evaluated for variation in the total RFO concentration and composition in this study. Siginificant variation in RFO concentration and composition was observed in both domesticated and wild lentil genotypes. It is possible that additional natural variation may be found in lentil germplasm held by ICARDA that was not screened in our study. To facilitate the use of entire ICARDA lentil germplasm collection, the development of a representative minicore collection is needed. Development and evaluation of mutant lentil populations may also provide genetic resources as in the case of soybean for lentil quality improvement. In the RFO biosynthetic pathway, a regulatory enzyme has not been identified and our understanding of the function of several enzymes in the RFO and galactosyl cyclitols pathway is limited. The role of galactosyl cyclitols as donors of galactosyl residues for the formation of RFO is also not well understood. Similarly,

the role of galactinol besides RFO pathway if any is limited. Filling these gaps in our knowledge might, therefore, be critical for understanding RFO biosynthesis and accumulation in plants.

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APPENDICES

Appendix 3.1. Analyses of variance for different lentil traits.

Source	df	Mean Square							
		Protein	Starch					Weight	
			Starch	Amylose fraction	Hulled seeds	Dehulled seeds	Hulls	Seeds dehulled	Seed (1000)
Total	43								
Genotype	21	3.82**	4.24*	2.70*	4.35*	5.16*	0.003^{ns}	0.00^{ns}	266.62**
Replication	1	1.57^{ns}	1.53^{ns}	0.26^{ns}	1.47^{ns}	0.00^{ns}	0.004^{ns}	0.00^{ns}	0.53^{ns}
Residual	21	0.015	0.73	0.15	0.74	0.87	0.003	0.00	0.16
CV		0.47	2.0	1.21	1.97	1.83	114	1.91	1.15
R-square		0.99	0.85	0.95	0.86	0.86	0.51	0.60	0.99

** and * ns = Significant at < 0.01, < 0.05 and not significant, respectively.

Appendix 4.1. Analyses of variance and variance components of seed soluble carbohydrates.

Source	Df	Mean square		
		Glucose	Sucrose	Total RFO
Total	324[a] & 325[b]			
Environments	9	0.009**	2.255**	1.9**
Environments*Replication	20	0.001[ns]	0.020 [ns]	0.2 [ns]
Cultivar	10	0.004**	0.794**	3.0**
Cultivar*Environments	90	0.003**	0.067**	0.4**
Residual	195	0.001	0.009	0.2
$\sigma^2_{Environmental}$			0.6 (±0.3)	0.0 (± 0.0)
$\sigma^2_{Cultivar}$			0.2 (±0.1)	0.1 (±0.1)
$\sigma^2_{Cultivar\ s\times Environment}$			0.2 (±0.0)	0.1 (±0.0)
$\sigma^2_{Residual}$			0.2 (±0.0)	0.2 (±0.0)
CV		100.3	6.6	9.5
R-square		0.70	0.95	0.7
h^2			0.89	0.85

** Significant at < 0.01; [ns] not significant
[a] Glucose and sucrose; [b] Total RFO

Appendix 4.2. Analyses of variance of RFO composition in seeds

Source	Df	Mean Square		
		Raffinose	Stachyose	Verbascose
Total	316[a] & 315[b]			
Environment	9	1.90**	4.11**	0.30**
Environment*Replication	20	0.2[ns]	0.07[ns]	0.01[ns]
Cultivar	10	0.19**	0.26**	0.08**
Cultivar*Environment	90	0.06**	0.14**	0.02**
Residual	187[a] & 186[b]	0.03	0.05	0.01
CV		9.33	9.84	8.69
R-square		0.82	0.85	0.74

** Significant at < 0.01; [ns] not significant
[a] raffinose and stachyose, [b] Verbascose

Appendix 5.1. Sucrose, verbascose, stachyose and raffinose concentrations in domesticated lentil (*Lens culinaris ssp. culinaris*) seeds.

Country of origin	Province	Genotype	Sucrose (g 100 g^{-1}flour)	Total RFO (mmoles 100 g^{-1}flour)	Individual RFO (g 100 g^{-1}flour)		
					Raffinose	Stachyose	Verbascose
Afghanistan	Paravan	ILL 1762	1.6	5.1	2.3	2.8	1.4
Afghanistan	Farah	ILL 213	1.9	5.2	2.3	3	1.4
Afghanistan	Heart	ILL 7567	1.2	5.2	2.3	2.5	1.4
Afghanistan		ILL 2217	1.4	5.4	2.3	2.6	1.2
Argentina		ILL 4605	1.2	4.9	2.4	2.7	1.5
Armenia	Artik	ILL 86	2.2	4.9	2.5	2.8	1.3
Azerbaijan	Nachichevan	ILL 1671	1.8	5.4	2.2	2.7	1.3
Bangladesh	Gopalganj district	ILL 7773	0.7	5.3	2.3	2.5	1.4
Bulgaria		ILL 5399	0.6	5.0	2.5	2.9	1.4
Brazil		ILL 6967	1.4	5.1	2.3	2.3	1.2
Canada		ILL 4349	1.0	5.2	2.4	2.6	1.4
Chile		ILL 2290	1.0	5.2	2.5	2.8	1.4
Chile		ILL 5490	0.9	5.3	2.5	2.9	1.4
China		ILL 7499	0.9	5.6	2.5	2.9	1.2
Colombia		ILL 1649	1.3	5.7	2.4	3	1.2
Cyprus	Limassol	ILL 5968	1.2	5.1	2.4	2.8	1.3
Czech Republic		ILL 4783	1.0	5.4	2.4	2.7	1.3
Germany		ILL 4831	1.1	4.5	2.3	2.7	1.3
Algeria	Batna	ILL 7051	1.5	5.2	2.2	2.5	1.4
Egypt	Marsa Matruh	ILL 6540	1.8	4.3	2.2	2.6	1.3
Egypt		ILL 784	0.9	5.3	2.4	3.1	1.5
Egypt	El Giza	ILL 4387	1.4	5.5	2.3	2.7	1.2
Spain	Castilla la Mancha	ILL 80	1.1	5.1	2.6	3.1	1.5

Country of origin	Province	Genotype	Sucrose (g 100 g^{-1}flour)	Total RFO (mmoles 100 g^{-1}flour)	Individual RFO (g 100 g^{-1}flour)		
					Raffinose	Stachyose	Verbascose
Spain	Castilla la Mancha	ILL 5058	1.2	5.4	2.3	2.5	1.2
Ethiopia		ILL 1983	1.3	4.9	2.1	2.6	1.3
Ethiopia	Gonder	ILL 5945	1.0	5.0	2.4	2.7	1.4
Ethiopia	Sidama	ILL 2433	0.7	5.1	2.5	2.9	1.4
Ethiopia	Shewa	ILL 1683	1.1	5.4	2.5	2.9	1.4
Ethiopia	Shewa	ILL 1744	0.8	5.6	2.3	3.0	1.5
France	Center	ILL 4740	0.7	5.2	2.6	3.1	1.2
Greece	Peloponnesus	ILL 4865	1.0	4.5	2.5	2.7	1.3
Greece	Ionian Islands	ILL 293	1.0	4.9	2.6	3.1	1.3
Guatemala		ILL 494	0.9	4.9	2.4	2.9	1.3
Croatia	Croatia	ILL 4915	0.5	5.3	2.3	2.6	1.3
Hungary		ILL 4665	1.2	5.3	2.4	2.7	1.3
India		ILL 4359	0.8	4.1	2.3	2.5	1.3
India		ILL 3596	1.3	4.4	1.9	2.0	1.2
India		ILL 2684	1.2	4.5	2.2	2.4	1.3
India		ILL 3597	1.1	4.5	1.9	2.0	1.2
India		ILL 2501	1.1	4.6	2.4	2.6	1.3
India		ILL 2789	1.2	4.6	2.2	2.4	1.3
India		ILL 3805	1.1	4.6	1.9	2.0	1.2
India		ILL 2581	1.2	4.7	2.3	2.5	1.3
India		ILL 3347	1.2	4.7	1.9	2.0	1.2
India		ILL 4080	1.1	4.7	2.2	2.3	1.2
India		ILL 2526	1.3	4.8	2.1	2.3	1.2
India		ILL 3925	1.0	4.8	2.5	2.7	1.3
India		ILL 2607	1.1	4.9	1.7	2.0	1.2
India		ILL 3025	0.9	4.9	2.3	2.5	1.2
India		ILL 3714	1.1	4.9	1.7	1.9	1.1
India	Uttar Pradesh	ILL 132	1.4	5.1	2.6	3.0	1.3

Country of origin	Province	Genotype	Sucrose (g 100 g^{-1}flour)	Total RFO (mmoles 100 g^{-1}flour)	Individual RFO (g 100 g^{-1}flour)		
					Raffinose	Stachyose	Verbascose
India		ILL 4164	0.9	5.1	2.5	2.9	1.4
India	West Bengal	ILL 5151	1.0	5.1	2.4	2.7	1.3
India		ILL 3167	1.1	5.2	1.8	2.0	1.2
India	West Bengal	ILL 5080	0.7	5.6	2.5	2.9	1.4
Iran		ILL 2372	1.5	4.9	2.3	2.6	1.3
Iran	Esfahan	ILL 1553	2.0	5.0	1.7	2.0	1.2
Iran	Fars	ILL 242	1.4	5.2	2.4	2.9	1.3
Iran	Mazandaran	ILL 871	1.9	5.4	2.5	3.2	1.4
Iran	Kerman	ILL 1462	1.3	5.4	2.0	2.5	1.3
Iran	Gilan	ILL 4903	1.5	5.4	2.2	2.6	1.3
Iran	East Azerbaijan	ILL 7621	1.3	5.4	2.3	2.5	1.3
Iran	Khorasan	ILL 1337	1.7	5.6	2.3	2.9	1.4
Iran	Fars	ILL 1048	1.5	5.8	2.2	2.8	1.2
Iran	Fars	ILL 1220	1.4	6.1	2.3	2.9	1.3
Iraq	Dahuk	ILL 55	1.1	4.7	2.5	2.9	1.4
Italy	Puglia	ILL 5418	0.9	4.8	2.5	2.7	1.3
Jordan		ILL 5883	0.9	5.0	2.4	2.8	1.4
Jordan	Ma'an	ILL 6920	1.1	5.0	2.5	2.7	1.4
Jordan	Karak	ILL 9	1.3	5.2	2.5	3.0	1.6
Jordan	Karak	ILL 5584	1.2	5.2	2.5	2.8	1.5
Jordan	Irbid	ILL 5209	1.1	5.3	2.5	2.9	1.4
Lebanon		ILL 1139	1.8	5.3	1.7	2.4	1.2
Libya		ILL 4804	1.0	4.9	2.4	2.9	1.3
Morocco	Centre Nord	ILL 6505	1.1	4.8	2.4	2.5	1.3
Morocco	Centre	ILL 7727	1.1	4.8	2.3	2.4	1.4
Mexico		ILL 5425	0.9	4.7	2.7	2.7	1.3
Mecedonia	Macedonia	ILL 624	1.0	4.9	2.5	3.0	1.4
Netherlands		ILL 4609	0.9	4.7	2.3	2.4	1.3
Norway		ILL 4782	1.3	5.2	2.5	2.7	1.3

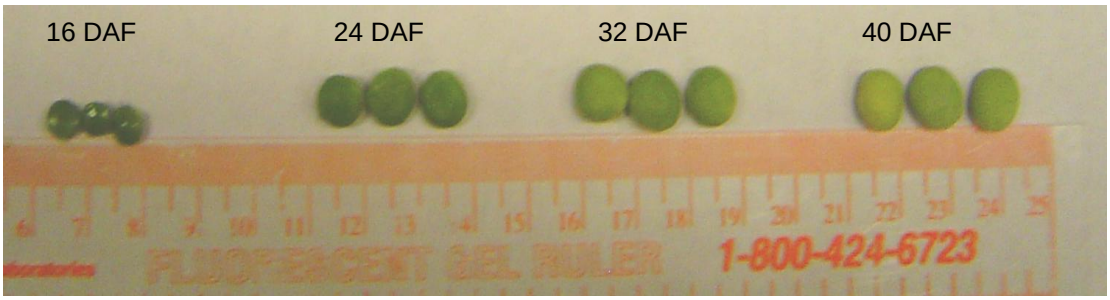
Country of origin	Province	Genotype	Sucrose (g 100 g^{-1}flour)	Total RFO (mmoles 100 g^{-1}flour)	Individual RFO (g 100 g^{-1}flour)		
					Raffinose	Stachyose	Verbascose
Nepal	Narayani	ILL 7791	1.1	4.4	2.2	2.2	1.2
Nepal	Janakpur	ILL 3487	1.3	4.5	1.5	1.5	1.1
Nepal	Janakpur	ILL 7314	1.2	5.9	2.3	2.7	1.2
Pakistan	NWF	ILL 6378	1.6	4.8	2.4	2.4	1.3
Pakistan	NWF	ILL 229	1.0	5.1	2.4	3.0	1.3
Pakistan	Northern Areas	ILL 2194	1.4	5.7	2.4	2.8	1.2
Palestine	Haifa	ILL 313	0.9	5.0	2.9	3.5	1.4
Poland	Lublin	ILL 5424	0.6	4.5	3.0	2.8	1.3
Portugal		ILL 4956	0.7	4.6	2.3	2.5	1.3
Romania		ILL 4774	1.1	4.8	2.4	2.7	1.3
Russia	Tambov oblast	ILL 7089	0.9	4.2	2.3	2.3	1.3
Russia		ILL 2191	1.1	4.7	2.6	2.8	1.4
Saudia Arabia	Al-Baha, Aseer	ILL 7745	1.1	4.7	2.1	2.2	1.3
Serbia	Sebia	ILL 5576	1.2	4.6	2.2	2.4	1.3
Sudan	Al Khartum	ILL 1861	1.0	5.2	2.3	3.0	1.5
Slovakia		ILL 4785	1.0	4.3	2.4	2.5	1.3
Syria	Aleppo	ILL 7747	1.0	4.8	2.0	2.1	1.2
Syria	Al Hasakah	ILL 5511	0.9	4.9	2.4	2.6	1.4
Syria	Al Qunaytirah	ILL 6689	0.9	4.9	2.5	2.9	1.6
Syria	Lattakia	ILL 6853	1.3	4.9	2.5	2.5	1.3
Syria	Homs	ILL 28	1.3	5.0	2.6	3.1	1.5
Syria	Al Hasakah	ILL 4542	1.0	5.0	2.5	2.7	1.4
Syria		ILL 4400	1.3	5.3	2.6	2.8	1.5
Tajikistan		ILL 618	1.7	4.9	2.2	2.6	1.3
Tunisia		ILL 6182	0.8	4.4	2.3	2.5	1.3
Turkey	Nigde	ILL 7585	1.1	4.5	2.3	2.5	1.3
Turkey	Tunceli	ILL 141	1.5	4.7	2.5	3	1.3
Turkey	Hakkari	ILL 2171	1.0	4.9	2.2	2.5	1.3
Turkey	Kastamonu	ILL 927	2.7	5.1	2.1	2.8	1.2

Country of origin	Province	Genotype	Sucrose (g 100 g^{-1}flour)	Total RFO (mmoles 100 g^{-1}flour)	Individual RFO (g 100 g^{-1}flour)		
					Raffinose	Stachyose	Verbascose
Turkey	Corum	ILL 6166	1.4	5.2	2.3	2.5	1.2
Turkey	Tunceli	ILL 572	1.6	5.8	2.3	2.8	1.1
United Kingdom	Kiev	ILL 595	1.2	5.1	2.4	3	1.4
UnKnown		ILL 3502	1.1	5.0	1.8	1.9	1.1
Uruguay		ILL 4778	0.5	4.0	2.7	2.7	1.3
USA		ILL 4671	0.8	5.1	2.3	2.7	1.4
Uzbekistan		ILL 4875	1.2	5.2	2.5	3	1.4
Yemen	San'a	ILL 4768	1.3	4.7	2.4	2.6	1.4
Yugoslavia		ILL 2230	1.0	5.1	2.3	2.7	1.3
		ILL 5845	1.0	5.3	2.7	2.8	1.4
		ILL 6264	1.1	5.1	2.3	2.7	1.4
		ILL 9901	1.1	4.9	2.4	2.6	1.3
		ILL 10021	0.9	4.9	2.0	2.2	1.3
Std Error			0.11 - 0.15	0.19 - 0.26	0.14 - 0.17	0.17 - 0.21	0.05 - 0.06
Range			0.6 - 2.7	4.0 - 6.1	1.5 - 3.0	1.5 - 3.5	1.1 - 1.6

Appendix 5.2. RFO and sucrose concentrations in wild lentil seeds.

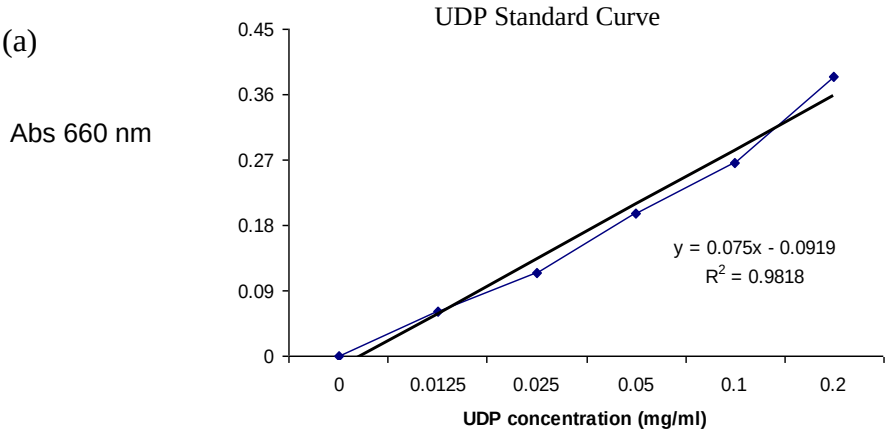
Latin name	Country of origin	Genotype	Sucrose (g 100 g^{-1}flour)	TRFO (mmoles 100 g^{-1} flour)	Verbascose	Stachyose (g 100 g^{-1}flour)	Raffinose
L. odemensis	Turkey	ILWL 202	1.7	2.9	1.0	1.8	2.2
L. odemensis	Syria	ILWL 174	0.7	4.9	0.9	2.1	2.1
L. odemensis	Palestine	ILWL 20	0.9	5.2	0.8	2.0	1.9
		Range	0.7 - 1.7	2.9 - 5.2	0.8 - 1.0	1.8 - 2.1	1.9 - 2.2
L. c. ssp. orientalis	Uzbekistan	ILWL 212	0.7	2.8	0.8	2.1	1.8
L. c. ssp. orientalis	Tajikistan	ILWL 381	1.2	3.4	0.8	1.7	2.0
L. c. ssp. orientalis	Syria	ILWL 330	0.5	3.6	0.9	2.1	2.2
L. c. ssp. orientalis	Turkmenistan	ILWL 380	0.8	4.0	0.8	2.6	2.6
L. c. ssp. orientalis	Jordan	ILWL 335	0.8	4.1	0.8	1.8	2.0
L. c. ssp. orientalis	Cyprus	ILWL 73	1.4	4.3	0.8	1.8	2.1
L. c. ssp. orientalis	Turkey	ILWL 4	0.4	4.6	0.8	2.3	2.2
L. c. ssp. orientalis	Iran	ILWL 70	0.9	4.7	0.8	2.6	2.5
		Range	0.7 - 1.4	2.8 - 4.7	0.8 - 0.9	1.7 - 2.6	1.8 - 2.6
L. tomentosus	Syria	ILWL 149	0.4	3.7	0.9	2.3	2.5
L. tomentosus	Turkey	ILWL 91	0.7	4.1	0.5	2.1	2.5
		Range	0.4 - 0.7	3.7 - 4.1	0.5 - 0.9	2.1 - 2.3	2.5
L. ervoides	Turkey	ILWL 285	0.2	1.7	1.4	1.8	1.8
L. ervoides	Ukraine	ILWL 328	0.3	2.0	1.4	1.8	1.8
L. ervoides	Syria	ILWL 412	0.2	2.0	1.3	1.6	2.0
L. ervoides	Italy	ILWL 42	0.1	2.1	1.4	1.8	1.9
L. ervoides	Azerbaijan	ILWL 0	0.2	2.1	1.4	1.8	1.9
L. ervoides	Slovenia	ILWL 44	0.2	2.2	1.5	1.8	1.9
L. ervoides	Palestine	ILWL 55	0.2	2.4	1.3	1.7	1.7
L. ervoides	Turkey	ILWL 262	0.2	2.5	1.5	1.7	2.0
L. ervoides	Syria	ILWL 155	0.3	2.6	1.5	1.8	1.8
L. ervoides	Armenia	ILWL 0	0.3	2.8	1.2	1.8	2.3
L. ervoides	Bosnia	ILWL 0	0.3	2.8	1.3	1.6	1.6

Latin name	Country of origin	Genotype	Sucrose (g 100 g^{-1}flour)	TRFO (mmoles 100 g^{-1} flour)	Verbascose	Stachyose (g 100 g^{-1}flour)	Raffinose
L. ervoides	Yugoslavia	ILWL 0	0.3	2.9	1.7	2.2	2.2
L. ervoides	Turkey	ILWL 461	0.4	2.9	1.3	1.7	1.9
L. ervoides	Syria	ILWL 133	0.3	2.9	1.2	1.5	1.5
L. ervoides	Crotia	ILWL 52	0.3	2.9	1.3	1.6	1.9
L. ervoides	Syria	ILWL 298	0.3	2.9	1.5	2.0	2.0
L. ervoides	Turky	ILWL 62	0.3	3.0	1.4	1.7	1.7
L. ervoides	Serbia	ILWL 51	0.4	3.1	1.5	1.9	1.9
L. ervoides	Jordan	ILWL 339	0.4	3.1	1.4	1.7	1.6
L. ervoides	Lebanon	ILWL 394	0.4	3.4	1.5	1.9	1.9
		Range	0.1 - 0.4	1.7 - 3.4	1.2 - 1.7	1.5 - 2.2	1.5 - 2.3
L. lamottei	France	ILWL 14	0.4	2.2	1.0	1.6	2.0
L. lamottei	Spain	ILWL 432	0.5	2.5	0.9	1.9	2.3
L. lamottei	Turkey	ILWL 437	0.6	3.2	0.9	1.8	2.1
		Range	0.4 - 0.6	2.2 - 3.2	0.9 - 1.0	1.6 - 1.9	2.0 - 2.3
L. nigricans	Ukraine	ILWL 327	0.4	2.4	0.3	1.8	2.8
L. nigricans	Italy	ILWL 0	0.4	2.5	0.8	1.8	2.4
L. nigricans	Crotia	ILWL 0	0.3	2.5	0.8	1.9	2.5
L. nigricans	Serbia	ILWL 0	0.6	2.5	0.8	1.6	2.3
L. nigricans	Spain	ILWL 0	0.4	2.6	0.8	2.1	3.0
L. nigricans	Bosnia	ILWL 0	0.3	2.7	0.9	1.8	2.5
L. nigricans	Turkey	ILWL 474	0.7	3.0	0.8	1.8	2.5
L. nigricans	Turkey	ILWL 112	0.9	3.4	0.8	1.8	2.2
L. nigricans	Turkey	ILWL 453	0.5	3.4	0.9	2.1	2.3
		Range	0.3 - 0.9	2.4 - 3.4	0.3 - 0.9	1.6 - 2.1	2.2 - 3.0
Std Error			0.1 - 0.2	0.3 - 0.5	0.1	0.1 - 0.2	0.1 - 0.2
Overall Range			0.1 - 1.7	1.7 - 5.2	0.3 - 1.7	1.5 - 2.6	1.5 - 3.0

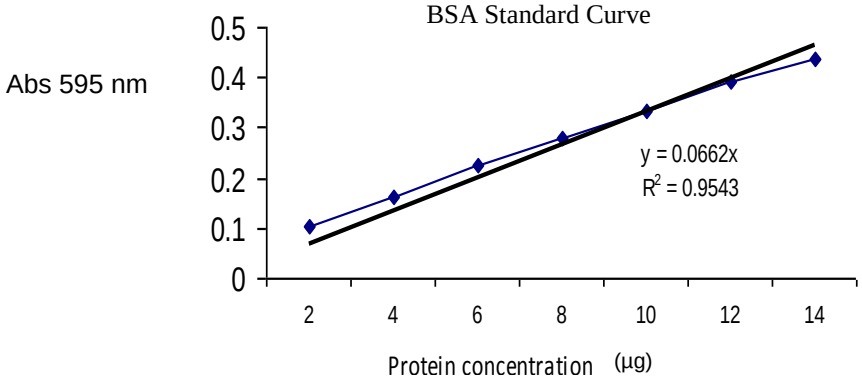


Appendix 6.1. Lentil seeds at different days after flowering (DAF).

(a)

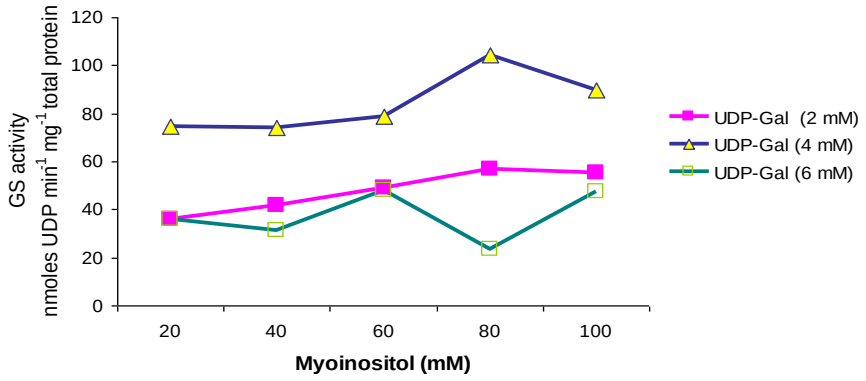


(b)

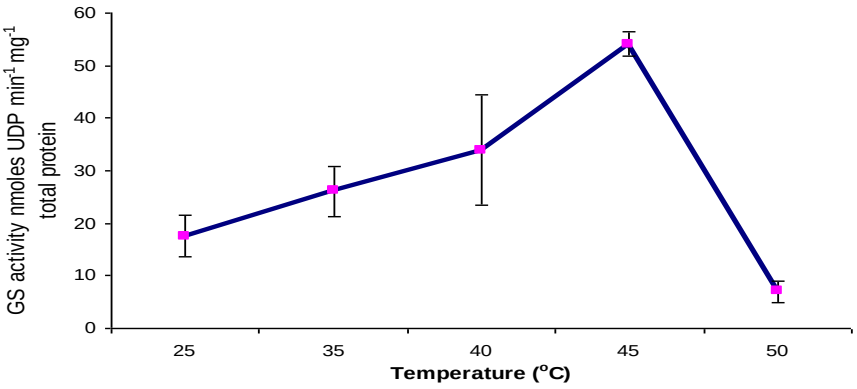


Appendix 6.2. Standard curves for (a) inorganic Pi and (b) protein determination. The darker linear lines indicate the calculated linear regression.

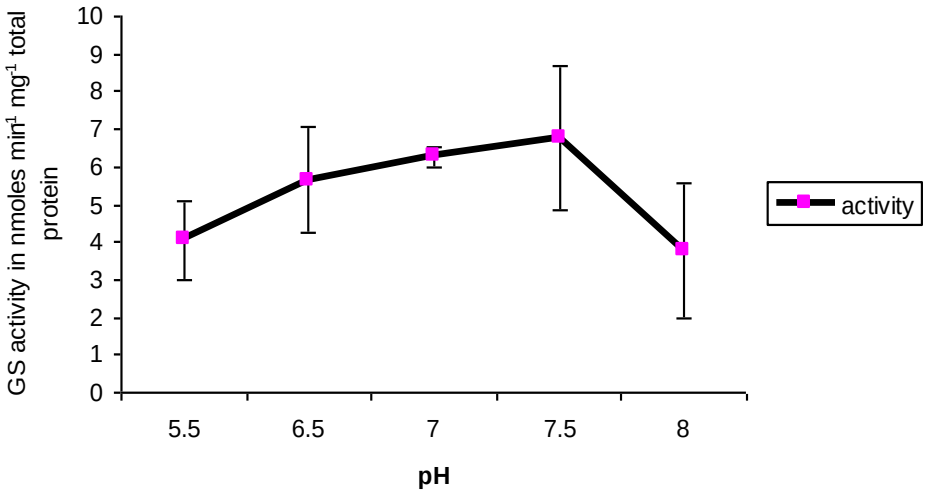
(a)



(b)

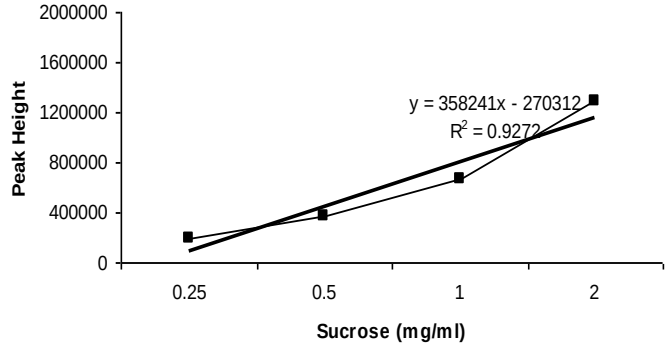


(c)

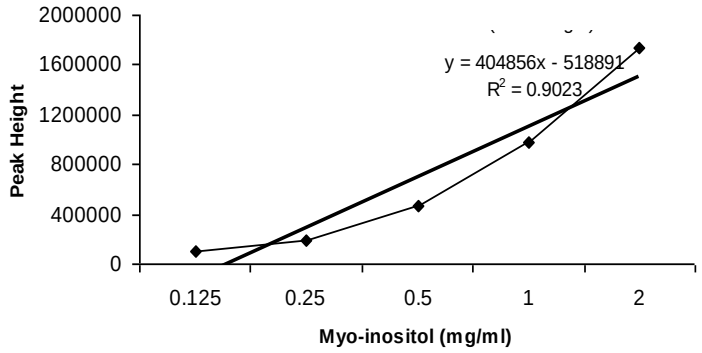


Appendix 6.3. GS activity assayed at (a) different UDP-galactose and myo-inositol concentrations (b) at different temperatures and (c) at different pH.

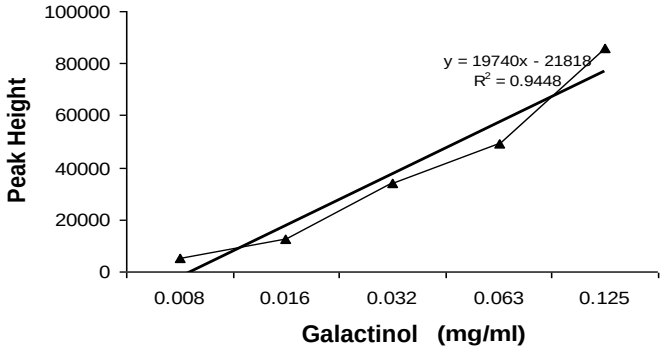
(a)



(b)



(c)



Appendix 6.4. Standard curves for (a) sucrose (b) myo-inositol and (c) galactinol determination.